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(57) Abstract

The present invention provides a peptide compound of formula Z^1-K-A-Q-X^1-K-K-N-K-H-R-H-S-X^2-T-Z^2 [SEQ ID No. 1] where: X^1 represents S or Y, X^2 represents T or I, Z^1 represents the N terminus of the peptide, or from 1 to 47 amino acids, Z^2 represents the C terminus of the peptide, a terminal amide group, or from 1 to 77 amino acids; or a variant of this peptide which contains one or more internal deletions, insertions or substitutions and which substantially retains anti-coagulant properties of apoB-100.

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ANTICOAGULANT PEPTIDE FRAGMENTS DERIVED FROM APOLIPOPROTEIN B-100

The present invention relates to peptides with anticoagulant properties. These are useful in a number of clinical applications, for example counteracting hypercoagulable states and in surgical procedures.

Background to the invention.

The extrinsic pathway of coagulation is of central importance to the formation of thrombin in major blood vessels. This is initiated by the exposure of tissue factor (thromboplastin) from endothelial cells and monocyte/macrophages in the arterial wall.

Antidotes to tissue factor (TF), such as tissue factor pathway inhibitor, are found in the circulation associated with plasma proteins. However we have also found that plasma lipoproteins, particularly low density lipoproteins, can also exert an inhibitory effect on tissue factor activity (Ettelaie et al, 1995, Biochim. Biophys. Acta. 1257; 25-30).

The inhibition of TF is caused by the apolipoprotein B-100 (apoB-100). Its action is slower, but more long lasting than that of TFPI.

The exposure of thromboplastin, upon injury to endothelium, initiates the extrinsic pathway of coagulation and leads to formation of a blood clot on the injured surface [1,2]. Following clot formation the procoagulant effect of thromboplastin is restrained by a number of circulating inhibitors within the serum. The ability of low density lipoprotein (LDL) [3-5] to inhibit the procoagulant activity of thromboplastin has been demonstrated previously. We have demonstrated that this inhibition arises from direct interaction of the protein moieties of thromboplastin (apoprotein III) and LDL (apolipoprotein B-100) [5-7]. Furthermore, the binding of the proteins involves the positively charged residues within apolipoprotein B-100 and

negatively charged amino acids on thromboplastin [7]. We have previously identified a region within thromboplastin which closely resembled the repeated domains within the LDL-receptor protein, that are responsible for binding to apolipoprotein B-100 [7].

Inhibition of thromboplastin by apo B-100 may be disrupted with poly-L-lysine and to a lesser extent by poly-L-arginine [7]. Furthermore, blocking of lysine residues, e.g. during oxidation also seems to disrupt the inhibition [4,5].

Brief Description of the drawings

Fig. 1. Time-course assay of the inhibition of thromboplastin by KRAD-14, KRAD-98 and reconstituted apolipoprotein B-100.

Fig. 2. Effect of the KRAD-14 concentration on thromboplastin inhibition.

Fig. 3a. Top view of the four-helix bundle model of KRAD-98 peptide.

Fig. 3b. Stereo model of the interaction of KRAD-14 with thromboplastin fragment (58-66).

Fig. 3c. Space-filling model of KRAD-14 peptide and thromboplastin fragment (56-66) indicating the interacting amino acids.

Fig. 4. Effect of the KRAD-14 peptide concentration on inhibition of thromboplastin.

Fig. 5. Time-course assay of the inhibition of the procoagulant activity of factor X by KRAD-14 peptide.

Fig. 6. Time-course assay of the inhibition of the procoagulant activity of factor V by KRAD-14 peptide.

Fig. 7. Influence of the peptide 58-66, on the interaction and inhibitiion of thromboplastin by apo B-100.

Disclosure of the invention.

By cloning and expression of a fusion protein containing the lysine-rich apolipoprotein B-100 derived (KRAD-14) peptide (14 amino acids long), we have explored the potential involvement of this domain in the interaction and inhibition of thromboplastin by apolipoprotein B-100.

Although apoB-100 is a very large protein with a molecular weight of about 515 kDa, we have surprisingly found that a small region within the protein is responsible for the inhibitory effect on TF. We have found that a peptide with a sequence derived from this region has much higher activity than apoB-100 itself. This peptide sequence is active when used in as a free peptide and also when present in a larger 98 amino acid sequence derived from apoB-100.

The KRAD-14 peptide has been examined against other coagulation factors. It seems to have an inhibitory effect against prothrombinase complex (factor Xa and factor V) in activating thrombin. The KRAD-14 peptide also has an ability to prevent the activation factor VII on the surface of thromboplastin. Furthermore, the KRAD-14 peptide seems to affect the activation of platelets by thrombin, probably due to prevention of the activation of thrombin. However, direct effect of KRAD-14 peptide on platelet activation cannot be ruled out.

In addition, recombinant thromboplastin (residues 58-66) was prepared and its effect on thromboplastin activity and inhibition by apolipoprotein B-100 was examined. It was demonstrated that this peptide is involved in the interaction of thromboplastin with apolipoprotein B-100.

Thus in a first aspect the present invention provides a peptide compound of formula:

Z^1-K-A-Q-X^1-K-K-N-K-H-R-H-S-X^2-T-Z^2 [SEQ ID No. 1]

where:

X^1 represents S or Y,

X^2 represents T or I,

Z^1 represents the N terminus of the peptide, or from 1 to 47 amino acids,

Z^2 represents the C terminus of the peptide, a terminal amide group, or from 1 to 77 amino acids;

or a variant of this peptide which contains one or more internal deletions, insertions or substitutions and which substantially retains anti-coagulant properties of apoB-100.

In the above formula the peptide sequence is represented by the standard 1-letter code.

The compound may be in the form of a salt, since it will comprise a number of acidic and basic side chains as well as, usually, a carboxy terminus.

The invention further provides a pharmaceutical composition comprising a compound of the formula (I) together with a pharmaceutically acceptable diluent or carrier.

The invention also provides a compound or composition of the invention for use in a method of treatment or therapy of the human or animal body. Such treatment includes the various treatments described below.

The invention also provides a method of treatment of hypercoagulable states which comprises administering to a patient in need of treatment an effective amount of the compound or composition according to the invention.

The invention also provides a method of reducing the risk of formation of undesired blood coagulation during a surgical procedure performed on a human or animal subject which comprises administering to the subject an effective amount of the compound

or composition of the invention during surgery or post-operatively.

It is also known that some tumours secrete thromboplastin which is believed to assist in the process of metastasis. Prevention of metastasis is a major object of cancer therapy since a significant proportion of cancer deaths are attributable not to the primary tumour but to its metastatic spread. Thus in yet another aspect the present invention provides a method of reducing or preventing the process of metastasis in a human or animal subject suffering from a primary tumour which comprises administering to a patient in need of treatment an effective amount of the compound or composition according to the invention.

In this process, the compound or composition of the invention may be used in combination with other anti-metastatic compounds. Particular examples of such compounds includes matrix metalloproteinase inhibitors. Thus in a further aspect the invention provides a composition comprising a compound of the invention and a matrix metalloproteinase inhibitor.

The invention additionally comprises products containing a compound of the invention and a matrix metalloproteinase inhibitor as a combined preparation for simultaneous, separate or sequential use in cancer therapy.

Detailed description of the invention.

A. Compounds of Formula (I).

A.(i) When the N terminus of the compound of formula (I) shown as Z^1 is not simply the N terminal of the peptide it may comprise any suitable stretch of amino acids which allow the compound to retain anti-coagulant activity at least substantially similar to that found in apoB-100. The suitable stretch may comprise from 1 to 47, eg from 1 to 30, 1 to 20, or from 1 to 5 amino acids. The stretch of amino acids may be derived from apoB-100, for example they may represent the amino acids immediately to the N-terminal region of the 14 amino acids set

out in Seq. ID No. 1. These N-terminal amino acids are set out as amino acids 1 to 47 in Seq. ID No. 12.

The region Z^1 may alternatively or in addition comprise a leader sequence. Such a sequence may be present when for example the compound of the invention is produced by recombinant means. In such a case the leader sequence will be selected to be compatible with the host cell in which the compound is produced. The leader sequence may for example direct expression of the compound through the cell wall.

The leader sequence may also contain a sequence susceptible of cleavage to release a peptide compound of the invention. Suitable leader sequences include the sequence DDDDK which is an enterokinase cleavage site. This may be placed in front of the sequence of the peptide of the invention when the peptide is produced by recombinant means.

A further possible option is that the group Z^1 represents methionine.

A.(ii) When the C terminus of the compound of formula (I) shown as "Z^2" is not simply the C terminal of the peptide (i.e. a carboxy group) it may comprise any suitable stretch of amino acids which allow the compound to retain anti-coagulant activity at least substantially similar to that found in apoB-100. The suitable stretch may comprise from 1 to 77, eg from 1 to 30, 1 to 20, 1 to 10 or from 1 to 5 amino acids. The stretch of amino acids may be derived from apoB-100, for example they may represent the amino acids immediately to the C-terminal region of the 14 amino acids set out in Seq. ID No. 1. These N-terminal amino acids are set out as amino acids 62 to 138 in Seq. ID No. 2.

The region Z^2 may alternatively or in addition comprise a signal sequence. Such a sequence may be present when for example the compound of the invention is produced by recombinant means. In such a case the signal sequence will be selected to be compatible with the host cell in which the compound is produced. The signal

sequence may for example direct expression of the compound through the cell wall or to a particular cellular compartment.

The leader sequence may also contain a sequence susceptible of cleavage to release a compound of formula (I). Suitable cleavage sites include the sequences mentioned above in section A(i).

Preferably, Z^2 represents a carboxy group.

A.(iii) Peptides of the invention include fragments of Seq. ID No. 1, particularly fragments which retain the motif KKNK, preferably X^1KKNKH where X^1 is as defined above.

Thus for example fragments of the peptide of Seq. ID No. 1 include:

Q-X^1-K-K-N-K-H-R	[SEQ ID No. 2]
A-Q-X^1-K-K-N-K-H	[SEQ ID No. 3]
A-Q-X^1-K-K-N-K-H-R	[SEQ ID No. 4]
Q-X^1-K-K-N-K-H-R-H	[SEQ ID No. 5]
A-Q-X^1-K-K-N-K-H-R-H	[SEQ ID No. 6]
Q-X^1-K-K-N-K-H-R-H-S	[SEQ ID No. 7]
A-Q-X^1-K-K-N-K-H-R-H-S	[SEQ ID No. 8]
K-A-Q-X^1-K-K-N-K-H-R-H-S	[SEQ ID No. 9]
A-Q-X^1-K-K-N-K-H-R-H-S-X^2	[SEQ ID No. 10]
K-A-Q-X^1-K-K-N-K-H-R-H-S-X^2	[SEQ ID No. 11]

Variants of fragments include substitutions of one or more (e.g up to 4) amino acids. Preferred substitutions are conservative substitutions. Substitutions of this type may be made for a variety of reasons. For example, as demonstrated below, the residues X^1 and X^2 in apoB-100 are normally Y and I respectively, but these have been changed to S and T respectively to increase the solubility of the peptide. Similar types of changes may be made to the other amino acids of the peptide where this does not alter the activity to any significant degree.

Conservative substitutions may be made according to the following table, where amino acids on the same block in the second column

and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y
OTHER		N Q D E

A.(iv) Insertions to the sequence of formula (I) may also be made. One or more amino acids (e.g. up to 4) may be inserted at any position in the sequence of the compound. Preferably, any insertions made will be selected to avoid major changes to tertiary structure. This can be calculated using commercially available algorithms and tested empirically using methods described herein.

A.(v) Where the peptide of the invention comprises N- and/or C-terminal extensions such extensions are desirably based upon the sequence of apoB-100 itself.

Such peptides are up to 138 amino acids in size, and comprise all of all part (as defined below) of the sequence:

```
1          11         21         31         41         51
NIPLTIPEMR LPYTIITTPP LKDESLWEKT GLKEFLKTTK QSFDLSVKAQ YKKNKHRHSI

61         71         81         91         101        111
TNPLAVLCEF ISQSIKSFDR HFEKNRNNAL DFVTKSYNET KIKFDKYKAE KSHDELPRTF

121        131
QIPGYTVPVV NVEVSPFT
```

[SEQ ID No. 12]

where Y at position 51 may also be S and I at position 60 may also be T.

Where part of the sequence of Seq. ID No. 12 is referred to, it will be understood that such a portion of the sequence will comprise the motif KKNK, preferably X^1KKNKH where X^1 is as defined above, and more preferably any one of the sequences set out above as Seq ID Nos. 2-11.

A preferred portion of the peptide of Seq. ID No. 2 is the 98 amino acid sequence from position 21 (L) to 118 (R).

However, portions of any contiguous 15, e.g 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 or 120 amino acids of the sequence may be used provided such portions retain the KKNK motif as described above.

It will also be understood that modifications to the sequence of Seq ID No. 12 may be made provided such modifications do not substantially alter the activity of such a sequence or portions thereof compared to the unaltered sequence or portion thereof respectively. Such modifications include one or more (e.g. up to about 15% of the number of total residues in the peptide) substitutions, particularly conservative substitutions as defined above, and/or one or more (e.g. up to about 15% of the number of total residues in the peptide) deletions.

Computer modelling suggests that, within apolipoprotein B-100, the domain including KRAD-98 and KRAD-14 (amino acids 3121-3217) is structurally closely associated with another positively charged sequence, in the region of amino acids 3300-3400. More specifically, the positively charged sequence is believed to be RLTRKRGLKLAT (SEQ ID No. 13), which is the sequence of amino acids 3359-3367 of apolipoprotein B-100 (see below) Therefore, this sequence may be able to enhance the activity of the peptides of SEQ ID No.1.

Therefore, in preferred embodiments of the invention, a first peptide of SEQ ID No. 1, or a variant thereof as defined herein,

is associated with a second peptide which comprises amino acids 3300-3400 of apolipoprotein B-100, or a part or variant thereof as defined below. The two peptides may be physically attached in any way. For example, the second peptide may be provided as an N-terminal or C-terminal extension of the peptide of SEQ ID No.1, joined by a peptide bond, or it may be attached in some other way, for example by one or more covalent linkages, such as cysteine-cysteine disulphide linkages. The attachment can be made by means known in the art.

Alternatively, the second peptide may be physically unattached to the peptide of SEQ ID No. 1 but administered together with it. Thus, the invention provides pharmaceutical compositions comprising peptides of SEQ ID No. 1 and a second peptide which comprises amino acids 3300-3400 of apolipoprotein B-100, or a variant thereof as defined below, together with a pharmaceutically acceptable carrier.

The second peptide may include the entire sequence of amino acids 3300-3400 of apolipoprotein B-100. Most preferably, the second peptide consists of amino acids 3300-3400 of apolipoprotein B-100. However, the second peptide may have more or less than the sequence of amino acids 3300-3400 of apolipoprotein B-100, or have a sequence differing from that of amino acids 3300-3400 of apolipoprotein B-100 as long as the second peptide enhances the activity of the peptide of SEQ ID No. 1. For example, the second peptide may contain one or more internal deletions, insertions or substitutions, as defined herein for the peptides of SEQ ID No. 1.

Similarly, the second peptide may be extended, in the sense that it may contain extensions at one or both ends. These extensions may be the sequences that are adjacent to the second peptide in apolipoprotein B-100 or any other sequence. The extension or extensions may be of any length, for example 1 to 10, 10 to 20, or 20 to 50 amino acids.

Similarly, the second peptide may be truncated with respect to amino acids 3300-3400, as long as it retains the ability to

enhance the activity of the first peptide. For example, the truncated second peptide may be up to 20, up to 50, up to 80, up to 90, up to 95 or up to 99 amino acids in length.

More particularly, the second peptide may comprise the positively charged sequence, SEQ ID No. 13. For example, the second peptide may have exactly the sequence of SEQ ID No. 13 or it may be contain one or more deletions, insertions or substitutions as defined herein for the peptide of SEQ ID No. 1. Similarly, it may be extended or truncated as defined above as long as it retains the ability to enhance the activity of the first peptide.

In the sequence of apolipoprotein B-100 (see below), both KRAD-14 and the sequence of SEQ ID No. 13 are underlined. Also, two cysteine residues (3167 and 3397) are underlined. Where the first and second peptides are joined by cysteine-cysteine covalent linkages, these two cysteines may conveniently be used. Alternatively, if the particular peptides being used do not include both of those cysteine residues, other means of attachment may be used. In particular, additional cysteine residues may be incorporated into the first and/or second peptides to facilitate their attachment. This can be done by routine techniques of peptide synthesis already known in the art.

A.(vi) Peptides of the invention and the variants described above may be made by any suitable means available to those of skill in the art. The compounds may be made synthetically or by recombinant means.

Synthetic production of peptides is well known in the art and is available commercially from a variety of companies. Recombinant production may be achieved via the use of a nucleic acid (preferably DNA) encoding the sequence of the compound of formula (I) operably linked to a promoter for the expression of the said DNA and optionally a regulator of the promoter. This construct will be placed in a vector which may be, for example, a plasmid, virus or phage vector provided with an origin of replication. The vector may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial

plasmid or a neomycin resistance gene for a mammalian vector. The vector may be used to transfect or transform a host cell. Suitable cloning and expression vectors are available commercially and can be used for this purpose.

Vectors comprising nucleic acid encoding the peptides of the invention themselves form a further aspect of the invention.

A further embodiment of the invention provides host cells transformed or transfected with the vectors of the invention for the replication and expression of vectors according to the invention. The cells will be chosen to be compatible with the vector and may for example be bacterial, yeast, insect or mammalian.

Conditions for the expression of foreign proteins in expression vectors are well known in the art and may be utilized to express a compound of the invention in the host cell and recover said compound in purified form.

A.(vii) The amino acid sequence of the peptides of the invention may also be modified to include non-naturally-occurring amino acids or to increase the stability of the compound *in vivo*. When the compounds are produced by synthetic means, such amino acids may be introduced during production. The compound may also be modified following either synthetic or recombinant production.

Peptides of the invention which are of a size suitable for synthetic production may also be made using D-amino acids. In such cases, the amino acids will be linked in a reverse sequence in the C to N orientation. This is conventional in the art for producing such peptides.

The carboxy terminus and any other carboxy side chains may be blocked in the form of an ester group, e.g. a C_{1-6} alkyl ester.

Salts of compounds of the invention which may be conveniently used in therapy include physiologically acceptable base salts, eg derived from an appropriate base, such as alkali metal (e.g.

sodium), alkaline earth metal (e.g. magnesium) salts, ammonium and NR_4 (wherein R is C_{1-4} alkyl) salts. Salts also include physiologically acceptable acid addition salts, including the hydrochloride and acetate salts.

A.(viii) As mentioned above, variants of the compounds of the formula (I) which are included within the scope of the invention are those which substantially retain the anti-coagulant properties of apoB-100. This may be determined by making such variants and assaying them by methods described in the examples for their ability to inhibit the activity of thrombin. Variants which show activity which is at least 0.1 fold, preferably 0.5 fold, the activity of apoB-100 are considered as retaining the necessary anti-coagulant activity. This may be determined by any suitable assay, for example the thromboplastin activity assay illustrated in the accompanying examples.

B. Compositions of the invention.

B.(i) Pharmaceutically acceptable carriers or diluents include those used in formulations suitable for oral or parenteral (e.g. intramuscular or intravenous) administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

For example, formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents, and liposomes or other microparticulate systems which are

designed to target the polypeptide to blood components or one or more organs.

Suitable liposomes include, for example, those comprising the positively charged lipid (N[1-(2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA), those comprising dioleoylphosphatidyl-ethanolamine (DOPE), and those comprising 3β[N-(n',N'-dimethyl-aminoethane)-carbamoyl]cholesterol (DC-Chol).

Optionally, the peptides of the invention may be attached to drug delivery substrates in order to facilitate their delivery to the correct location in the patient. Suitable substrates include polymeric beads known in the art. Such beads may, preferably, be engineered to target the peptides of the invention to a given location in the body.

C. Treatment of hypercoagulable states.

There are a number or medical conditions where patients are at risk from blood clotting which may benefit from administration to the patient of a peptide of the invention. These include patients suffering from chronic cardiovascular conditions as well as acute thrombotic episodes, e.g heart attacks or strokes. Administration of a peptide of the invention to such patients will help reduce the risk of such conditions or their recurrence following a thrombotic episode.

D. Treatment during surgical procedures.

There are many types of surgical procedure where the risk of blood coagulation during surgery or post operatively may be reduced by use of the compound of the invention. Such procedures include coronary reperfusion, transplantation of organs (e.g. liver, kidney, heart or lung transplantations), and major surgery such as open heart surgery.

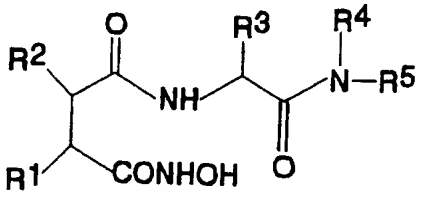
E. Prevention or reduction of metastasis.

Metastases are associated with almost every type of cancers. These include colon, ovarian, breast, prostate, lung (including small cell lung), stomach and liver cancers. Preventing or reducing metastatic spread represents a major goal of current cancer research. Reducing the spread includes both reducing the number of metastases which are established in a patient as well as reducing the rate at which metastases grow by invasion of normal tissue.

All the above-mentioned tumour types are candidates for treatment with compounds of the invention for the prevention or reduction of metastatic spread. Preferred tumour types are those where thromboplastin is being expressed by the primary tumour.

The peptide compounds of the invention may be administered in conjunction with other anti-tumour agents, particularly agents which also prevent or inhibit metastatic spread. Suitable other anti-metastatic compounds include matrix metalloproteinase (MMP) inhibitors.

Such inhibitors are well known in the art and include hydroxamic acid based MMP inhibitors of the general structure:



wherein the five substituents R^1 to R^5 may vary. Examples of such compounds may be found in several patent publications including US-A-4599361, EP-A-231081, EP-A-236872, EP-A-274453, WO90/05716, WO90/05719, WO91/02716, WO92/09563, EP-A-497192, WO92/13831, EP-A-489577, EP-A-489579, WO92/22523, US-A-6256657, WO93/09090, WO93/09097, WO93/20047, WO93/21942, WO93/24449, WO93/24475, EP-A-

574758, WO94/02446, WO94/02447 and WO94/21625, the disclosures of which are incorporated herein by reference.

Suitable values of the group R^1 include hydrogen, C_{1-6} alkyl, C_{2-6} alkenyl, phenyl, phenyl C_{1-6} alkyl, C_{1-6} alkylthiomethyl, phenylthiomethyl, phenyl C_{1-6} alkylthiomethyl or hetrocyclylthiomethyl where hetrocyclyl is as defined below.

Suitable values of R^2 include a C_2-C_6 alkyl group which may contain an ether or thioether linkage;

Suitable values of R^3 include (a) the side chain of a naturally occurring alpha-amino acid in which any carboxylic acid groups may be esterified or amidated, any hydroxyl or thiol groups may be acylated or alkylated (etherified) and any amino groups may be acylated, or (b) a group $R^6(A)_n$- wherein n is 0 or 1, A represents a divalent C_1-C_6 alkyl or C_2-C_6 alkenyl group optionally interrupted by one or more -O-, or -S- atoms or -N(R^7)- groups where R^7 is hydrogen or C_1-C_6 alkyl, and R^6 is a phenyl or heterocyclyl group either of which may be substituted, or (except where n is 0) a hydrogen atom.

Suitable values of R^4 include hydrogen or methyl.

Suitable values of R^5 include hydrogen, C_1-C_6 alkyl or phenyl (C_1-C_6 alkyl).

The term "C_1-C_6 alkyl" refers to a straight or branched chain alkyl moiety including for example, methyl, ethyl, propyl, isopropyl and isobutyl.

Groups which may contain an ether or thioether linkage include $CH_3(CH_2)_2OCH_2$-, and $CH_3(CH_2)_2S$-.

The term "C_2-C_6 alkenyl" refers to a straight or branched chain alkenyl moiety having from 2 to 6 carbon atoms and having in addition one double bond of either E or Z stereochemistry where applicable.

The term "side chain of a naturally occurring alpha-amino acid" includes the side chains of the twenty naturally occurring amino acids encoded by the genetic code as well as naturally occurring substances such as 5-hydroxylysine and 4-hydroxyproline. The amino acid side chains may be protected; for example the carboxyl groups of aspartic acid and glutamic acid may be esterified (for example as a C_1-C_6 alkyl ester), the amino groups of lysine, 5-hydroxylysine, 4-hydroxyproline may be converted to amides (for example as a COC_1-C_6 alkyl amide) or carbamates (for example as a $C(=O)OC_1$-C_6 alkyl or $C(=O)OCH_2Ph$ carbamate).

The term "heterocyclyl" refers to a 5-7 membered heterocyclic ring containing one or more (e.g. 2) heteroatoms selected from S, N and O, and optionally fused to benzene ring, including for example, pyrrolyl, furyl, thienyl, imidazolyl, pyridinyl, pyrrolidinyl, pyrimidinyl, morpholinyl, piperizinyl and indolyl.

MMPs may be used in the form of salts including physiologically acceptable acid addition salts for example hydrochlorides, hydrobromides, sulphates, methane sulphonates, p-toluenesulphonates, phosphates, acetates, citrates, succinates, lactates, tartrates, fumarates and maleates, Salts may also be formed with bases, for example sodium, potassium, magnesium, and calcium salts.

There are several chiral centres in the MMP compounds because of the presence of asymmetric carbon atoms. The presence of several asymmetric carbon atoms gives rise to a number of diastereomers with R or S stereochemistry at each chiral centre. Unless specified otherwise all other formulae in this specification are to be understood to include all such stereoisomers and mixtures (for example racemic mixtures) thereof. The preferred stereochemistry is in general as follows:

C atom carrying the R^1 group and hydroxamic acid moiety -S,
C atom carrying the R^2 group -R,
C atom carrying the R^3 group -S,
but mixtures in which the above configurations predominate may also be used.

A particular example of such a compound is [4-N-hydroxyamino)-2R-isobutyl-3S-(thienyl-thiomethyl)succinyl]-L-phenylalanine-N-methylamide (known as Batimastat or "BB-94").

MMPs may be administered by any suitable route and in any formulation, for example those comprising a liquid or solid diluent or carrier. Oral administration, for example in tablet form, is one suitable route. Doses will be at the discretion of the physician depending upon the properties of the particular MMP and the nature of the condition being treated. Suitable doses include those in the range of from 0.1 to 300, e.g from 1 to 100 mg/kg body weight per day.

F. Prevention of other thromboplastin-related phenomena.

The inhibitory effect of the KRAD-14 peptide against thromboplastin may result in the prevention of other processes related to thromboplastin e.g. angiogenesis, cellular differentiation and apoptosis. Thus, the peptides of the invention may also be useful in the inhibition of these processes, and other (100 thromboplastin-related processes.

G. Routes of administration and doses of peptides of the invention.

G.(i) Suitable routes of administration of peptides of the invention include oral or parenteral, and will depend in part upon the intended use and the discretion of the physician. Small peptides may be administered orally although parenteral administration may generally be more convenient in some circumstances. For example, during surgical procedures the compounds may be administered via drips used to deliver blood and/or saline during surgery.

G. (ii) Although the amount of peptides of the invention administered to a patient is ultimately at the discretion of the physician, taking account of the condition of the patient and the condition to be treated, typical amounts of compounds of the invention required to achieve an anti-coagulant effect will be

effective to deliver a concentration of peptide in the region of from 0.1 μM to 10 mM, e.g from 1 μM to 1 mM in the body of a patient. This may be achieved using doses of about from 0.1 mg to 10g, eg. 1mg to 1g per Kg body weight per day.

The following examples illustrate the invention.

Materials and Methods.

Oligonucleotide synthesis was carried out on a Biosearch Cyclone oligonucleotide synthesiser (PerSeptive Biosystems Inc., Biosearch Products, U.K.) using reagents purchased from Cambio Ltd (Cambridge, U.K.). Pinpoint Xa protein expression kit, Wizard PCR preps kit, Wizard miniprep kit, Chromatophor protein visualisation system, streptavidin-alkaline phosphatase conjugate, Hind III and Not I restriction enzymes, T4 DNA ligase, Taq polymerase, *E.coli* (JM103) cells, nitro blue tetrazolium and bromochloroindoyl phosphate were obtained from Promega Corporation, Southampton, U.K.). Deoxynucleotides and ampicillin were from Gibco Life Sciences (Paisley, Scotland) and enterokinase, chymotrypsin, N-benzoyl-phe-val-arg-p-nitroanilide (for the two stage amidolytic assay), isopropyl-β-thiogalactopyranoside, biotin and Terrific broth were obtained from Sigma Chemical Company Ltd. (Poole, U.K.). The apolipoprotein B-100 control peptide (fragment 3358-3372) containing the sequence TRLTRKRGLKLATAL was also purchased from Sigma Chemical Company Ltd. (Poole, U.K.). Protean II electrophoresis and blotting apparatus was from Bio Rad (Hemel Hempstead, U.K.). Recombinant thromboplastin was from Gamidor Ltd. (Oxfordshire, U.K.). Control plasma (NormTrol), for the one-stage prothrombin time assay, was purchased from Helena Laboratories (Tyne and Wear, U.K.). A synthetic peptide with a sequence identical to that of chymotrypsin-released KRAD-14 was purchased to order, from the in-house peptide synthesis facility.

Cloning and expression of peptides

In order to minimise the possibility of peptide aggregation due to presence of hydrophobic amino acids the peptide sequence (KAQSKKNKHRHSTT (Seq. ID No. 1)) was prepared instead of the parent (KAQYKKNKHRHSIT (Seq. ID No. 1)). The secondary structure of the peptide was not compromised by these substitutions as determined by prediction methods [17,18]. Both the coding and non-coding strands were synthesised on the oligonucleotide synthesiser. The coding region was preceded by a codon for a single tyrosine to define a cleavage site for chymotrypsin. The oligonucleotides were prepared to encode a Hind III site at the 5' end of the coding site and a Not I site at the 3' end, as shown below;

5' AGCTTTATAA AGCTCAGTCC AAAAAAAACA AACACCGTCA CTCCACCACC TAAGC 3'
AATATT TCGAGTCAGG TTTTTTTTGT TTGTGGCAGT GAGGTGGTGG ATTCGCCGG

Plasmid Pinpoint Xa(3) was digested with Hind III and Not I overnight at 4°C, and an aliquot was examined on agarose gel in order to confirm complete digestion. The excised fragment was removed using the Wizard PCR preps kit. Following deprotection and isolation, the KRAD-14 oligonucleotide insert was ligated into Pinpoint plasmid using T4 DNA ligase at 4°C overnight.

KRAD-98 peptide (98 amino acids) corresponded to residues 3121-3217 of apolipoprotein B-100 sequence and residues 21 to 118 of Seq. ID No. 12. Overlapping strands of DNA (each 200 bp long) with non-identical termini, to enable unidirectional cloning, were engineered using the oligonucleotide synthesiser. An enterokinase cleavage site was included immediately preceding the apolipoprotein B-100-peptide sequence, this site is later used to release the biotinylated protein. Following deprotection and isolation, the two strands were heated together at 95°C for 1 min and annealed by progressive cooling down to 50°C. The strands were completed by addition of Taq polymerase (3 units) and 0.2 mM nucleotide solution. The product was examined by agarose gel electrophoresis. The KRAD-98 DNA was then digested with Hind III and Not I overnight at 4°C.

Similarly, the DNA sequence (shown below) coding for thromboplastin residues 58-66 (DLTDEIVKD) was prepared and cloned and the peptide expressed and isolated as described.

5' AGCTTTATGA CCTGACCGAC GAAATCGTGA AAGACTAAGC 3'
AATACT GGACTGGCTG CTTTAGCACT TTCTGATTCG CCGG

Competent *E. coli* (JM109) cells were subsequently transfected with either, the plasmid containing the KRAD-14 insert, KRAD-98 insert, thromboplastin fragment (58-66) insert, plasmid without any insert or with Pinpoint control plasmid (containing the chloramphenicol acetyltransferase gene as a fusion peptide) and selected by growth in Terrific broth, containing ampicillin (100 μg/ml). The cells were then grown in the presence of 100 μg/ml isopropyl-β-thiogalactopyranoside (IPTG) and 5 μg/ml biotin. The cells were harvested by centrifugation and washed in isolation buffer (50 mM Tris-HCl, 2 mM EDTA pH 7.8). The cells were then lysed by sonication in isolation buffer. The biotinylated proteins were isolated using the Softlink matrix (supplied with the pinpoint protein expression kit) according to the manufacturer's instructions. The peptide samples were then dialysed and freeze-dried.

Western blotting

SDS-polyacrylamide electrophoresis was carried out using a Protean II apparatus. A 12 % (w/v) acrylamide gel was prepared using the Chromatophor protein visualisation system and according to the manufacturer's instructions. The gel was then blotted onto nitrocellulose, blocked for 60 min with Tris buffer, saline Tween (TBST) (10 mM Tris-HCl pH. 8.0, 150 mM NaCl, 0.05% w/v Tween 20) and incubated for 30 min with streptavidin-alkaline phosphatase conjugate in TBST. The membrane was then washed three times with TBST and once with distilled water. Finally, the membrane was probed with nitro blue tetrazolium/ bromochloroindolyl phosphate (NBT/BCIP).

Release of the biotinylated tag using chymotrypsin or enterokinase

In order to release the KRAD-14 peptide from the biotinylated fusion protein, aliquots (0.5 mg/ml) of the isolated fusion protein were incubated with bovine pancreatic chymotrypsin (0.1 mg/ml final concentration). To release KRAD-98, aliquots (0.5 mg/ml) of the isolated fusion protein were incubated with enterokinase (0.1 mg/ml final concentration). In addition an equal volume of distilled water was treated with the same amount of chymotrypsin or enterokinase to ensure that at the concentrations used, the enzymes did not have a significant effect on the thromboplastin activity. The biotinylated tag was then removed by chromatography using the Softlink column as before. The digested samples were then examined on a 15 % (w/v) SDS-acrylamide gel to ensure proper release of the protein, as before and the bulk of the sample freeze dried.

Isolation and reconstitution of apolipoprotein B-100

Low density lipoproteins (LDL) were prepared by ultracentrifugation [19] and apolipoprotein B-100 was isolated by delipidation and gel filtration and reconstituted (1 mg/ml) in phosphatidylcholine (I mg/ml) as described previously [5, 19-21].

Measurement of thromboplastin activity

The one stage prothrombin time assay was carried out by the addition of 100 μl of the sample to 100 μl of 25 mM $CaCl_2$ solution and incubation prior to addition of 100 μl of control plasma. The clotting time was measured on the coagulometer. The two-stage amidolytic assay was carried according to published procedures [9]. In all cases the thromboplastin activity was calculated from a standard curve prepared previously and the percentage inhibition was calculated as 100 x (Initial activity/Residual activity)/ Initial activity.

Measurement of the effects of apolipoprotein B-100-derived peptides on thromboplastin activity

The apolipoprotein B-100 control peptide (fragment 3358-3372) containing the sequence TRLTRKRGLKLATAL was reconstituted in

distilled water to 1 mM. The freeze-dried samples were reconstituted in distilled water to a concentration of 1 mM. Two 200 µl samples of thromboplastin (10 U/ml) were incubated with 500 nM KRAD-14 or apolipoprotein B-100 control peptide at 37°C for 45 min. Another set of 200 µl samples of thromboplastin (10 U/ml) were incubated with either 500 nM KRAD-98 or the chloramphenicol acetyltransferase control protein. Finally, another 200 µl of thromboplastin was incubated with 500 nM reconstituted apolipoprotein B-100 [5] or equal amount of distilled water. Following the incubation the thromboplastin activity in all the samples was measured by means of the one-stage prothrombin time assay and the two-stage amidolytic assay and the percentage inhibition calculated against the control containing distilled water.

The above experiments were repeated using 1 ml samples containing recombinant thromboplastin (10 U/ml) mixed with either 500 nM KRAD-14, 500 nM KRAD-98 or 500 nM apolipoprotein B-100, reconstituted as described before [5]. The concentrations used are equivalent to that of apolipoprotein B-100 in normal plasma [8]. 100 µl of the each sample was removed immediately and assayed for thromboplastin activity, by means of the one-stage prothrombin time assay. The rest of the samples were incubated at 37°C and further 100 µl samples were removed at intervals up to 120 min and measured as before. The percentage inhibition was calculated against a control sample of thromboplastin.

In order to monitor the inhibitory potential of the isolated peptides in the presence of plasma, aliquots (100 µl) of the recombinant thromboplastin (10 U/ml) were incubated for 5 min, with equal amounts of either KRAD-14 or for 40 min with equal volumes of either KRAD-98 in the presence and absence of plasma, at 37°C. The samples were then assayed by addition of 100 µl of 25 mM $CaCl_2$ solution and measurement on the coagulometer. The percentage inhibition in each case calculated against a similarly treated sample, containing thromboplastin only.

The synthetic KRAD-14 peptide was reconstituted in distilled water at 1 mM. Aliquots of recombinant thromboplastin were

incubated, at 37°C with a KRAD-14 at concentration range 10-5000 nM (final concentration) for 1 min prior to assaying using the one-stage prothrombin time assay. In addition, samples of thromboplastin (10 U/ml) were incubated with synthetic KRAD-14 at concentrations ranging from 1-100 μM (final concentration) prior to assaying using the one-stage prothrombin time assay.

In other experiments, recombinant thromboplastin (1 mg/ml) was delipidated with chloroform/methanol as described previously [5,19], to remove phosphatidylserine and other lipids and reconstituted with phosphatidylcholine (1 mg/ml) to differentiate between the effects of the prepared samples on the protein moiety of thromboplastin and any effects on the thromboplastin-associated phosphatidylserine. Aliquots of recombinant thromboplastin (1 mg/ml) were incubated with KRAD-14 peptide (500 nM), KRAD-98 (500 nM) peptide, reconstituted apolipoprotein B-100 (500 nM) or each of the control peptides (500 nM) for 30 min prior to assaying using the one-stage prothrombin time and the two-stage amidolytic assay. The percentage inhibition was calculated against the control sample.

Measurement of influence of KRAD-14 on thrombin activity

Samples (1 ml) of either KRAD-14 peptide (500 nM) or each of the control peptides (500 nM) were incubated with thrombin (100 nM) at 37°C and the thrombin activity measured at the beginning and at intervals up to 60 min as follows. The enzymatic activity of thrombin was measured by the addition of 100 μl aliquots to 0.45 ml of thrombin substrate (N-benzoyl-phe-val-arg-p-nitroanilide) (0.26 mM) which was then incubated at 37°C for exactly 10 min, before the addition of 0.45 ml of 20% (w/v) acetic acid. The absorption was measured at 405 nm against a thrombin (100 nM) control assayed similarly. The procoagulant activity of thrombin was measured by the addition of 100 μl of the sample to control plasma (100 μl) and 25 mM $CaCl_2$ solution and measuring the clotting time. A thrombin 100 nM) control was included in the assay against which comparisons were made.

Measurement of influence of KRAD-14 on factor Xa activity

Samples (1 ml) of either KRAD-14 peptide (500 nM) or the control peptide 500 nM) were incubated with pre-activated factor Xa (100 nM) at 37°C and the factor Xa activity measured at the beginning and at intervals up to 60 min as follows. The enzymatic activity of factor Xa was measured by the addition of 100 μl aliquots to 0.45 ml of thrombin substrate (N-benzoyl-ile-glu-gly-arg-p-nitroanilide) (0.26 mM) which was then incubated at 37°C for exactly 10 min, before the addition of 0.45 ml of 20 % (w/v) acetic acid. The absorption was measured at 405 nm against a factor Xa (100 nM) control assayed similarly. The procoagulant activity of factor Xa was measured by the addition of 100 μl of the sample to factor Xa-deficient plasma (100 μl) and 25nM $CACl_2$ solution and measuring the clotting time. A factor Xa (100 nM) control was included in the assay against which comparisons were made.

Measurement of influence of KRAD-14 on factor V activity

Samples (1 ml) of either KRAD-14 peptide (500 nM) or the control peptide (500 nM) were incubated with pre-activated factor V (100 nM) at 37°C and the factor V activity measured at the beginning and at intervals up to 60 min as follows. The procoagulant activity of factor V was measured by the addition of 100 μl of the sample to factor V-deficient plasma (100 μl) pre-activated by incubation (2 min) with thromboplastin (200 nM) in the presence of $CACl_2$ (25 mM), and measuring the clotting time. The activity was measured against a standard curve and the percentage inhibition calculated against the initial factor V activity. The curves are typical of data from three separate experiments.

Measurement of the effect of KRAD-14 on the activation and activity of factor VII

In order to assess whether the action of KRAD-14 peptide was directed to the proteolytic activity of factor VIIa/thromboplastin or the activation of factor VII by interaction with thromboplastin, first thromboplastin (200 nM) and factor VII (200 nM) were incubated for 10 min in the presence of 25 mM $CaCl_2$, prior to addition of KRAD-14 peptide and a

further 10 min incubation. The samples were then assayed by addition to factor VII deficient plasma and measuring the clotting time. Appropriate controls devoid of KRAD-14 peptide were included in each experiment.

Measurement of the effects of thromboplastin peptide (58-66) on thromboplastin activity and inhibition

The control peptide (VQGEESDNK) was reconstituted to 1mM in distilled water. The control peptide was similar but not identical to the thromboplastin (58-66), in size, charge and amino acid composition but with a different sequence. In order to assess the effect of the thromboplastin peptide (58-66) on the activity of thromboplastin, samples of recombinant thromboplastin (100 nM) were incubated for 5 min, with the isolated thromboplastin-peptide (1 μM), control peptide (1 μM) or distilled water at 37°C prior to assaying using the one-stage prothrombin time assay. The activity was calculated from a thromboplastin standard curve.

To demonstrate the ability of the peptide to influence the interaction of thromboplastin with apo B-100 samples of recombinant thromboplastin (100 nM) were incubated with reconstituted apo B-100. (250 nM final concentration) in the presence and absence of the isolated thromboplastin peptide (1 μM) or control peptide (1 μM), at 37°C. Aliquots (100 μl) were removed at the start and at various intervals up to 180 min and assayed by means of the one-stage prothrombin time assay. The residual activity of thromboplastin measured in the samples was compared to the initial activity, at time zero, and percentage inhibition calculated.

Computer modelling of the interaction of KRAD-14 with thromboplastin (residues 58-66)

A computer based search of the entire primary sequence of apolipoprotein B-100 was carried out to determine the concentration of positive residues and lysine residues within the protein sequence. In addition information gathered using

"Peptide structure" program (GCG, University of Wisconsin, USA) produced clues about the span and structure of these domains.

KRAD-14 sequence was constructed using the Alchemy III and energy minimisation carried out, until the energy gradient (the difference in energy between two iterations) was below 0.01. In order to explain a possible mode of interaction between KRAD-14 and the complementary domain within thromboplastin (residues 58-66) the KRAD-14 peptide, essentially, an α-helix, was positioned at an approximate angle of 20° [12] to thromboplastin (residue 58-66), hence indicating the interacting amino acids.

The structure of KRAD-98 was predicted to be a four-helix bundle as determined by the Threader program [22]. The helices and the turns within the structure were determined and the structure was then modelled using the Alchemy III program.

The results are discussed below with reference to the figures which provide the following data.

Fig. 1 shows the time-course assay of the inhibition of thromboplastin by KRAD-14, KRAD-98 and reconstituted apolipoprotein B-100.

Three 1 ml samples containing recombinant thromboplastin (10 U/ml) were mixed with 500 nM KRAD-14 (solid square), 500 nM KRAD-98 (open square) or 500 nM apolipoprotein B-100 (triangle), reconstituted as described before. The concentrations used are equivalent to that of apolipoprotein B-100 in normal plasma. 100 µl of the each sample was removed immediately and assayed for thromboplastin activity, by means of the one-stage prothrombin time assay. The rest of the samples were incubated at 37°C and further 100 µl samples were removed at intervals up to 120 min and measured as before. The thromboplastin activity was calculated from a standard curve prepared previously and the percentage inhibition was calculated as 100 x (Initial activity/Residual activity)/ Initial activity. The curves are typical of data from four sets of experiments.

Fig. 2 shows the effect of the KRAD-14 concentration on thromboplastin inhibition.

Aliquots of recombinant thromboplastin were incubated, at 37°C with a KRAD-14 at concentration range 10-5000 nM (final concentration) for 1 min prior to assaying using the one-stage prothrombin time assay. The percentage inhibition was calculated as 100 x (Initial activity/Residual activity)/ Initial activity. The experiment was performed in triplicate.

Fig. 3a Top view of the four-helix bundle model of KRAD-98 peptide.

KRAD-98 sequence (apolipoprotein B-100 residues 3121-3217) was constructed as a four-helix bundle using Alchemy III. The amino acids involved in the turns were omitted for clarity. The positively charged amino acids, lysine (K), arginine (R) and histidine (H) residues were highlighted to demonstrate the positive envelope surrounding the domain. KRAD-14 sequence has been indicated.

Fig. 3b shows a stereo model of the interaction of KRAD-14 with thromboplastin fragment (58-66).

Samples of thromboplastin (10 U/ml) were incubated with synthetic KRAD-14 concentration ranging 1-100 μM (final concentration) prior to assaying using the one-stage prothrombin time assay. The percentage inhibition was calculated as 100 x (Initial activity/Residual activity)/ Initial activity. The experiment was performed in triplicate.

KRAD-14 sequences were constructed using the Alchemy III and energy minimisation carried out, until the energy gradient was below 0.01. In order to explain a possible mode of interaction between KRAD-14 and the complementary domain within thromboplastin (residues 58-66) the KRAD-14 peptide, essentially, an α-helix, was positioned at an approximate angle of 20° to

thromboplastin (residue 58-66), hence indicating the interacting amino acids.

Fig. 3c. Space-filling model of KRAD-14 peptide and thromboplastin fragment (56-66) indicating the interacting amino acids.

KRAD-14 sequence were constructed using Alchemy III and energy minimisation carried out, until the energy gradient was below 0.01. In order to explain a possible mode of interaction between KRAD-14 peptide and the complementary domain within thromboplastin (residues 56-66) the KRAD-14 peptide, essentially, an alpha-helix, was positioned at an approximate angle of 20° to thromboplastin (residue 56-66), hence indicating the interacting amino acids. The interacting positive and negative residues were marked and the two sequence turned by 90° in opposite directions to reveal the interacting-charged pockets. In the model, aspartate 56 (thromboplastin) interacts with lysine 3147 (apo B-100), aspartate 58 (thromboplastin) interacts with lysine 3150 and 3151 (apo B-100), aspartate 61 (thromboplastin) interacts with arginine 3155 and histidine 3154 (apo B-100), glutamate 62 (thromboplastin) interacts with lysine 3153 and histidine 3156 (apo B-100).

Fig. 4. Effect of the KRAD-14 peptide concentration on inhibition of thromboplastin.

Aliquots (1 ml) of recombinant thromboplastin (100 nM) were incubated at 37°C with a KRAD-14 peptide at concentration range 50 (♦), 100 (▲), 200 (■), 300 (◇) and 500 nM (□) (final concentration). 100 µl of the each sample was removed immediately and assayed for thromboplastin activity, by means of the one-stage prothrombin time assay. The rest of the samples were incubated at 37°C and further 100 µl samples were removed at intervals up to 40 min and measured. The percentage inhibition was calculated as 100 x (Initial activity-Residual activity)/ Initial activity. The data were obtained from 3 separate experiments.

Fig 5. Time-course assay of the inhibition of the procoagulant activity of factor X by KRAD-14 peptide

Three 1 ml samples containing recombinant KRAD-14 peptide (20 (♦), 40 (▲) and 80 (■) nM) were mixed with factor Xa (100 nM). 100 µl of the each sample was removed immediately and assayed for factor Xa activity by addition to factor Xa-deficient plasma in the presence of $CaCl_2$ (25 mM) and measuring the clotting time. The rest of the samples were incubated at 37°C and further 100 µl samples were removed at intervals up to 40 min and measured as before. The activity was measured against a standard curve and the percentage inhibition calculated against the initial factor Xa activity. The curves are typical of data from 3 separate experiments.

Fig 6. Time-course assay of the inhibition of the procoagulant activity of factor V by KRAD-14 peptide.

Three 1 ml samples containing recombinant KRAD-14 peptide (20 (♦), 40 (▲) and 80 (■) nM) were mixed with factor V (1 µM). 100 µl of the each sample was removed immediately and assayed for factor Xa activity by addition to factor V-deficient plasma, pre-activated by incubation (2 min) with thromboplastin (200 nM) in the presence of $CaCl_2$ (25 mM), and measuring the clotting time. The rest of the samples were incubated at 37°C and further 100 µl samples were removed at intervals up to 40 min and measured as before. The activity was measured against a standard curve and the percentage inhibition calculated against the initial factor V activity. The curves are typical of data from 3 separate experiments.

Fig 7. Influence of the peptide 58-66, on the interaction and inhibition of thromboplastin by apo B-100

Samples of thromboplastin (100 nM) were incubated with reconstituted apo B-100 (250 nM final concentration) in the presence (■) and absence (▲) of the isolated thromboplastin peptide (1 µM), at 37°C. Aliquots (100 µM) were removed at the

start (time zero) and at various intervals up to 180 min and assayed by means of the one-stage prothrombin time assay. The data presented are representative of 5 independent experiments.

Results

Preparation of recombinant peptides

On propagation only the cells containing the control plasmid or the plasmid-insert survived. The procedure used allowed only the unidirectional insertion of the KRAD-14, KRAD-98, control peptides and thromboplastin (58-66) fragments. Western blotting of KRAD-14 and KRAD-98 peptides revealed bands of biotinylated protein with molecular weights of 15 KDa (thromboplastin fragment 58-66), 18 KDa (KRAD-14) and 27 KDa (KRAD-98) which agree with theoretical molecular weight calculated for the fusion peptides. The control chloramphenicol acetyltransferase sample contained a protein with a molecular weight of 40 KDa (control protein). In addition, fainter bands of biotinylated proteins (22.5 KDa) were detected, which are endogenous to the strains of *E. coli*.

Effect of KRAD-14 and KRAD-98 on thromboplastin activity

The data from the one-stage prothrombin assay and the two-stage amidolytic assay were comparable and the percentage inhibition was calculated from the data generated using both methods. In the first experiment, samples of thromboplastin (100 nM) were incubated with KRAD-14 peptide (500 nM), KRAD-98 peptide (500 nM), reconstituted apolipoprotein B-100 (500 nM) or each of the control peptides (500 nM) at 37°C. Samples were removed immediately and at intervals up to 120 min and assayed for thromboplastin activity. KRAD-14 inhibited the procoagulant activity of thromboplastin by 98 % following 4 min incubation. KRAD-98 exhibited a 95 % inhibition of thromboplastin after 45 min incubation. On the other hand, neither the control 15mer apolipoprotein B-100 (fragment 3358-3372) peptide nor the chloramphenicol acetyltransferase control protein had any detectable effect on thromboplastin. Reconstituted apolipoprotein B-100 also inhibited the procoagulant activity of thromboplastin

(>95 %) after 90 min incubation. The inclusion of calcium-free plasma during the incubations prior to assaying, did not significantly affect the ability of KRAD-14 or KRAD-98 peptide (500 nM) to inhibit thromboplastin activity.

On incubation of thromboplastin with KRAD-14 peptide, the procoagulant activity was inhibited rapidly, reaching 90 % at 2 min and a maximum of 98 % within 4 min (Fig 1). On the other hand, both KRAD-98 attained an inhibition level of >90 % following a minimum of 40 min incubation, and reconstituted apolipoprotein B-100 took 70 min to reach inhibition levels of >90%.

Preincubation of plasma with thromboplastin resulted in a slightly shorter clotting time and hence a higher apparent thromboplastin activity. However, the presence of plasma did not affect the ability of the KRAD-14 to inhibit thromboplastin activity and following 2 min incubation 74 % inhibition was detected. Similarly, KRAD-98 exhibited 95 % inhibition following 40 min incubation.

The incubation of thromboplastin with a range of KRAD-14 peptide concentrations (10-500 nM) demonstrated that the inhibition is concentration dependent and exhaustible (Fig 2). However, KRAD-14 was effective at concentration as low as 50 nM. Moreover, the inhibition was unstable and completely disappeared after approximately 8 min following reconstitution. This deterioration was partially stabilised by adjusting the pH and addition of 5 nM bovine serum albumin as a stabiliser. However, the inhibitory effect deteriorated rapidly, completely disappearing after 15 min, following reconstitution.

The preincubation of thromboplastin (100 nM) with a range of KRAD-14 peptide concentrations (1-5000 nM) for 1 min at 37°C, demonstrated that the inhibition is concentration dependent and exhaustible (Fig 4) ($Kd_{app}=3.7 \times 10^{-8}$). However KRAD-14 peptide was effective at concentrations as low as 50 nM. The synthetic KRAD-14 peptide was also inhibitory towards thromboplastin with similar effectiveness as the recombinant peptide.

Reconstitution of delipidated recombinant thromboplastin in phosphatidylcholine reduced the activity of the sample by 200 fold. However, the presence of KRAD-14 (500 nM) or KRAD-98 (500 nM) peptides inhibited this activity by over 99 and 85 % respectively (Table 1). This indicates that the inhibitory effect does not solely arise from the interaction of the KRAD-14 peptide with the negatively charged phosphatidylserine associated with thromboplastin.

Table 1. Inhibition of thromboplastin, reconstituted in phosphatidylcholine, by KRAD-14, KRAD-98 peptides and reconstituted apolipoprotein B-100.

Sample	Procoagulant Activity	% Inhibition
Distilled water (4.70 U/ml)	0.06	-
APO B-100 control peptide (3358-3372) (4.68 U/ml)	0.06	0.4
Reverse KRAD-14 (4.68 U/ml)	0.08	0.4
Proline-rich KRAD-14 (4.71 U/ml)	0.04	-0.2
Scrambled KRAD-14 (4.61 U/ml)	0.08	1.9
Recombinant KRAD-14 peptide (0.05 U/ml)	0.04	99
Recombinant KRAD-98 peptide (0.71 U/ml)	0.04	85
Reconstituted apo B-100 (1.79 U/ml)	0.05	62

Recombinant thromboplastin was delipidated to remove phosphatidylserine and other lipids and reconstituted at 1 mg/ml with phosphatidylcholine (1 mg/ml). Four 1 ml samples were incubated with either 500 nM KRAD-14 peptide, 500 nM KRAD-98 peptide, 500 nM reconstituted apolipoprotein B-100 or each of the four control peptides (500 nM) or distilled water at 37°C for 30 min, prior to assay. The % Inhibition was calculated against the control. The data were obtained from 3 separate experiments.

Effect of KRAD-14 on thrombin and factor Xa and factor V activity

The KRAD-14 peptide (500 nM) did not affect either the clotting ability or the amidolytic proteolytic activity of thrombin (100 nM). Neither control peptide had any significant effect on

thrombin activity. The peptide (500 nM) did not affect the amidolytic activity of factor Xa. However, some inhibition of coagulation was observed upon pre-incubation of KRAD-14 with either factor Xa (100 nM) (Kd_{app}= 2.75 x 10^{-7}) (Fig 5) or factor V (1 μM) (Kd_{app}= 2.85 x 10^{-7}) (Fig 6). The coagulation assays were carried out by incubating the KRAD-14 peptide with factor Xa or V respectively, at 37°C for up to 60 min, prior to assaying in factor Xa deficient plasma or preactivated factor V deficient plasma. Neither control peptide had any effect on the factor Xa or V activities.

Investigation of the effect of KRAD-14 on activation and activity of factor VII

In order to assess whether the action of KRAD-14 peptide was directed to the proteolytic activity of factor VIIa/thromboplastin or the activation of factor VII by interaction with thromboplastin, first thromboplastin (200 nM) and factor VII (200 nM) were incubated for 10 min in the presence of 25 mM $CaCl_2$, prior to addition of EPIC peptide (500 nM) and a further 10 min incubation. In the second experiment thromboplastin, factor VII and factor Xa (100 nM) were pre-incubated for 10 min in the presence of 25 mM $CaCl_2$, prior to addition of KRAD-14 peptide and a further 10 min incubation. The samples were then assayed by addition to factor VII deficient plasma and measuring the clotting time. KRAD-14 peptide did not significantly affect the activity of factor VIIa (inhibition = 12 % ± 8). Conversely, it was able to inhibit the activation of the pro-enzyme (inhibition = 74 % ± 8) (Table 2). Neither control peptide had any measurable effect on factor VII/thromboplastin activity or factor VII activation.

Table 2 The influence of peptide 58-66 on the procoagulant activity of thromboplastin.

Sample	Procoagulant Activity
Distilled water (6.70 U/ml)	**0.11**
Control peptide (6.75 U/ml)	**0.06**
Peptide 58-66 (7.45 U/ml)	**0.06**

Three aliquots of thromboplastin (100 nM) were incubated for 5 min, with the isolated thromboplastin peptide (1 μM), control peptide (1 μM) or distilled water at 37°C prior to assaying using the one-stage prothrombin time assay. The activity was calculated from a thromboplastin standard curve. The data were obtained from 3 separate experiments.

Effects of thromboplastin (58-66) on thromboplastin activity and inhibition

The isolated thromboplastin-derived peptide (58-66) had no endogenous procoagulant activity. The addition of the recombinant thromboplastin (1 μM) enhanced the procoagulant activity of thromboplastin (100 nM) while the control of peptide did not have any significant effect. Thromboplastin-peptide heterodimer exhibited a stronger procoagulant activity than the thromboplastin-thromboplastin homodimer. The presence of the thromboplastin peptide (1 μM) but not control peptide, also accelerated the rate of inhibition of thromboplastin activity (100 nM) by reconstituted apo B-100 (250 nM) (Fig 7). The thromboplastin peptide heterodimer exhibited a faster rate of interaction with apo B-100.

Computer modelling of the interaction of KRAD-14 with thromboplastin (residues 58-66)

The computer-based analysis of the entire apolipoprotein B-100 sequence revealed two distinct regions with a large proportion of positively charged amino acids. The sequences residues 3121-3217 (KRAD-98) and residues 3300-3400 are both within the receptor binding domain of apolipoprotein B-100. This is in agreement with published data [10-13]. The former domain is also especially rich in lysine residues. KRAD-98 was predicted to be a distinct four-helix bundle domain within apolipoprotein B100 (Fig 1). In this model the domain is surrounded by positively charged amino acids, particularly lysines, which make the domain hydrophilic and exposed. The highest concentration of lysine residues was within the amino acid regions 3147-3160 (KRAD-14). In order to explain a possible mode of interaction between KRAD-

14 and the complementary domain within thromboplastin (residues 58-66) the KRAD-14 sequence were constructed and positioned at an approximate angle of 20° [12] to thromboplastin (residue 58-66) (Fig. 3b). In this interaction, the lysine and histidine residues within KRAD-14 form pockets of positive charge around the negatively charged aspartate and glutamate amino acids within thromboplastin (residues 58-66).

In this model, the lysine 3150 and lysine 3151 of apo B-100, interact with aspartate 58 of thromboplastin, lysine 3153 and histidine 3156 of apo B-100, interact with aspartate 61 of thromboplastin and histidine 3154 and arginine 3155 of apo B-100, interact with glutamate 62 of thromboplastin. In addition, lysine 3147 of apo B-100, may interact with asparate 56 of thromboplastin.

The involvement of the thromboplastin sequence (58-66) and particularly aspartate 58 in binding and activity of factor VII has been demonstrated recently [13]. Hence, it is possible that, the interaction of KRAD-14 and apolipoprotein B-100 with this domain disrupts the ability of thromboplastin to bind factor VII, effectively inhibiting the procoagulant activity.

Discussion.

We previously reported that the interaction of thromboplastin with apolipoprotein B-100 occurs in at least two sites [7]. Thus, while the first site involves the attainment of a more hydrophobic configuration or the formation of hydrophobic interactions and occurs within 20 min of incubation, the evidence suggests that the second interaction, involves negatively charged residues and positive free amino groups, occurring over a longer period of time (up to 60 min). Furthermore, a possible apolipoprotein B-binding site on thromboplastin, due to its similarity to apolipoprotein B-100 binding domain within LDL-receptor protein, was indicated. In addition, it was reported that the interaction and inhibition of thromboplastin by apolipoprotein B is dependent on lysine residue within the primary sequence of the latter [Ettelaie et al unpublished data].

On the other hand, the presence of cationic amino acid per se was not sufficient for the inhibition since the cationic peptide from apolipoprotein B-100 (fragment 3358-3372) had no effect.. The ability of KRAD-98 to inhibit thromboplastin activity in the same manner as native apo B-100 suggests that the inhibitory potential of apo B-100 arises from this domain (residues 3121-3217). This domain is also believed to be involved in the interaction of apo B-100 with the cellular receptor for LDL [10], hence enforcing our previous suggestion that the interaction between apo B-100 and thromboplastin is essentially similar in nature to that between apo B-100 and its receptor [7]. In addition, previously, we suggested that the interaction and inhibition of thromboplastin by apolipoprotein B-100 occurs via a two-step mechanism [7], the first step of which involves the non-specific hydrophobic interaction of these two proteins. The absence of the lag time preceding the rise in inhibition, as observed with apo B-100 enforces this mechanism.

By attempting to match a complementary sequence to those found in thromboplastin (residues 58-66) and LDL-receptor protein (residues 283-291), we were able to select a sequence within apolipoprotein B-100 that fulfilled the criteria required. By cloning and expressing the peptide as a fusion protein, we were able to purify and assay any potential of this peptide sequence. The lysine-rich apolipoprotein B-100 derived (KRAD-14) peptide was altered in two amino acids to increase the hydrophilic property of the peptide without affecting its secondary structure, significantly.

The peptide sequence was shown to be an efficient inhibitor of thromboplastin. In addition, the rate of inhibition observed was much faster than that observed with either LDL or apolipoprotein B-100 [4-6,7,11]. This may be due to the immense size of apolipoprotein B and LDL since the protein has to manoeuvre into correct orientation for correct interaction with thromboplastin. Alternatively, the presence of lipids may be a factor in the rate of association of the two proteins. Also the inhibition was more stable than that observed with thromboplastin pathway inhibitor (TFPI) [11].

Thromboplastin is structurally related to class two cytokine receptors [14]. Furthermore, the peptide ligands for such receptors are normally helical [15]. Therefore, we propose that KRAD-14 is a suitable ligand for thromboplastin the binding of which masks amino acids essential for binding to factor VII and hence procoagulant activity.

The ability of these peptides to inhibit thromboplastin activity, when reconstituted in phosphatidylcholine, indicate that the activity of the peptide arises from a direct interaction with thromboplastin rather than a non-specific association with the negatively charged phosphatidylserine molecules which tend to enhance thromboplastin activity [2,27].

The thromboplastin (58-66) peptide enhanced the procoagulant activity of thromboplastin by acting co-operatively, an effect similar to that observed from the dimerisation of thromboplastin [27,28]. In addition, the smaller size of the peptide allows a faster rate of binding. This faster rate of interaction was also observed when examining the interaction of apo B-100 with thromboplastin. Therefore, the inhibitory action of apo B-100 on the extrinsic pathway of inhibition arises from the action on thromboplastin via direct interaction involving the sequence residues 58-66 within thromboplastin [8] and may displace factor VII preventing its activation.

This work has identified the interacting site between apolipoprotein B-100 and thromboplastin which results in the inhibition of the procoagulant activity of the latter. It appears that the interactions mask amino acids essential for binding and activation of factor VII, particularly aspartate 58 [22,23], hence inhibiting the procoagulant activity.

From this work we conclude that the inhibitory action of KRAD-14 or apolipoprotein B-100 on the extrinsic pathway of inhibition arises from a direct action on thromboplastin via direct interaction [7], and is dissimilar to that of TFPI which is essentially a protease inhibitor [16]. The accelerated effect of KRAD-14, as compared to apolipoprotein B-100, as well as its size

and ease of preparation, makes this a suitable potential agent in treatment of hypercoagulable conditions.

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SEQUENCE INFORMATION

Amino acid sequence of human apolipoprotein B-100.

KRAD-14 (amino acids 3147-3160) is underlined. The positively charged sequence within amino acids 3300-3400 (amino acids 3358-3367) is also underlined. Two cysteine residues that can be used to join the first and second peptides of the invention via a disulphide bond are also underlined.

1 EEEMLENVSL VCPKDATRFK HLRKYTYNYE AESSSGVPGT ADSRSATRIN

51 CKVELEVPQL CSFILKTSQC TLKEVYGFNP EGKALLKKTK NSEEFAAAMS

101 RYELKLAIPE GKQVFLYPEK DEPTYILNIK RGIISALLVP PETEEAKQVL

151 FLDTVYGNCS THFTVKTRKG NVATEISTER DLGQCDRFKP IRTGISPLAL

201 IKGMTRPLST LISSSQSCQY TLDAKRKHVA EAICKEQHLF LPFSYKNKYG

251 MVAQVTQTLK LEDTPKINSR FFGEGTKKMG LAFESTKSTS PPKQAEAVLK

301 TLQELKKLTI SEQNIQRANL FNKLVTELRG LSDEAVTSLL PQLIEVSSPI

351 TLQALVQCGQ PQCSTHILQW LKRVHANPLL IDVVTYLVAL IPEPSAQQLR

401 EIFNMARDQR SRATLYALSH AVNNYHKTNP TGTQELLDIA NYLMEQIQDD

451 CTGDEDYTYL ILRVIGNMGQ TMEQLTPELK SSILKCVQST KPSLMIQKAA

501 IQALRKMEPK DKDQEVLLQT FLDDASPGDK RLAAYLMLMR SPSQADINKI

551 VQILPWEQNE QVKNFVASHI ANILNSEELD IQDLKKLVKE VLKESQLPTV

601 MDFRKFSRNY QLYKSVSLPS LDPASAKIEG NLIFDPNNYL PKESMLKTTL

651 TAFGFASADL IEIGLEGKGF EPTLEALFGK QGFFPDSVNK ALYWVNGQVP

701 DGVSKVLVDH FGYTKDDKHE QDMVNGIMLS VEKLIKDLKS KEVPEARAYL

751 RILGEELGFA SLHDLQLLGK LLLMGARTLQ GIPQMIGEVI RKGSKNDFFL

801 HYIFMENAFE LPTGAGLQLQ ISSSGVIAPG AKAGVKLEVA NMQAELVAKP

851 SVSVEFVTNM GIIIPDFARS GVQMNTNFFH ESGLEAHVAL KAGKLKFIIP

901 SPKRPVKLLS GGNTLHLVST TKTEVIPPLI ENRQSWSVCK QVFPGLNYCT

951 SGAYSNASST DSASYYPLTG DTRLELELRP TGEIEQYSVS ATYELQREDR

1001 ALVDTLKFVT QAEGAKQTEA TMTFKYNRQS MTLSSEVQIP DFDVDLGTIL

1051 RVNDESTEGK TSYRLTLDIQ NKKITEVALM GHLSCDTKEE RKIKGVISIP

1101 RLQAEARSEI LAHWSPAKLL LQMDSSATAY GSTVSKRVAW HYDEEKIEFE

1151 WNTGTNVDTK KMTSNFPVDL SDYPKSLHMY ANRLLDHRVP QTDMTFRHVG

1201 SKLIVAMSSW LQKASGSLPY TQTLQDHLNS LKEFNLQNMG LPDFHIPENL

1251 FLKSDGRVKY TLNKNSLKIE IPLPFGGKSS RDLKMLETVR TPALHFKSVG

1301 FHLPSREFQV PTFTIPKLYQ LQVPLLGVLD LSTNVYSNLY NWSASYSGGN

1351 TSTDHFSLRA RYHMKADSVV DLLSYNVQGS GETTYDHKNT FTLSCDGSLR

1401 HKFLDSNIKF SHVEKLGNNP VSKGLLIFDA SSSWGPQMSA SVHLDSKKKQ

1451 HLFVKEVKID GQFRVSSFYA KGTYGLSCQR DPNTGRLNGE SNLRFNSSYL

1501 QGTNQITGRY EDGTLSLTST SDLQSGIIKN TASLKYENYE LTLKSDTNGK

1551 YKNFATSNKM DMTFSKQNAL LRSEYQADYE SLRFFSLLSG SLNSHGLELN

1601 ADILGTDKIN SGAHKATLRI GQDGISTSAT TNLKCSLLVL ENELNAELGL

1651 SGASMKLTTN GRFREHNAKF SLDGKAALTE LSLGSAYQAM ILGVDSKNIF

1701 NFKVSQEGLK LSNDMMGSYA EMKFDHTNSL NIAGLSLDFS SKLDNIYSSD

1751 KFYKQTVNLQ LQPYSLVTTL NSDLKYNALD LTNNGKLRLE PLKLHVAGNL

1801 KGAYQNNEIK HIYAISSAAL SASYKADTVA KVQGVEFSHR LNTDIAGLAS

1851 AIDMSTNYNS DSLHFSNVFR SVMAPFTMTI DAHTNGNGKL ALWGEHTGQL

1901 YSKFLLKAEP LAFTFSHDYK GSTSHHLVSR KSISAALEHK VSALLTPAEQ

1951 TGTWKLKTQF NNNEYSQDLD AYNTKDKIGV ELTGRTLADL TLLDSPIKVP

2001 LLLSEPINII DALEMRDAVE KPQEFTIVAF VKYDKNQDVH SINLPFFETL

2051 QEYFERNRQT IIVVLENVQR NLKHINIDQF VRKYRAALGK LPQQANDYLN

2101 SFNWERQVSH AKEKLTALTK KYRITENDIQ IALDDAKINF NEKLSQLQTY

2151 MIQFDQYIKD SYDLHDLKIA IANIIDEIIE KLKSLDEHYH IRVNLVKTIH

2201 DLHLFIENID FNKSGSSTAS WIQNVDTKYQ IRIQIQEKLQ QLKRHIQNID

2251 IQHLAGKLKQ HIEAIDVRVL LDQLGTTISF ERINDVLEHV KHFVINLIGD

2301 FEVAEKINAF RAKVHELIER YEVDQQIQVL MDKLVELAHQ YKLKETIQKL

2351 SNVLQQVKIK DYFEKLVGFI DDAVKKLNEL SFKTFIEDVN KFLDMLIKKL

2401 KSFDYHQFVD ETNDKIREVT QRLNGEIQAL ELPQKAEALK LFLEETKATV

2451 AVYLESLQDT KITLIINWLQ EALSSASLAH MKAKFRETLE DTRDRMYQMD

2501 IQQELQRYLS LVGQVYSTLV TYISDWWTLA AKNLTDFAEQ YSIQDWAKRM

2551 KALVEQGFTV PEIKTILGTM PAFEVSLQAL QKATFQTPDF IVPLTDLRIP

2601 SVQINFKDLK NIKIPSRFST PEFTILNTFH IPSFTIDFVE MKVKIIRTID

2651 QMLNSELQWP VPDIYLRDLK VEDIPLARIT LPDFRLPEIA IPEFIIPTLN

2701 LNDFQVPDLH IPEFQLPHIS HTIEVPTFGK LYSILKIQSP LFTLDANADI

2751 GNGTTSANEA GIAASITAKG ESKLEVLNFD FQANAQLSNP KINPLALKES

2801 VKFSSKYLRT EHGSEMLFFG NAIEGKSNTV ASLHTEKNTL ELSNGVIVKI

2851 NNQLTLDSNT KYFHKLNIPK LDFSSQADLR NEIKTLLKAG HIAWTSSGKG

2901 SWKWACPRFS DEGTHESQIS FTIEGPLTSF GLSNKINSKH LRVNQNLVYE

2951 SGSLNFSKLE IQSQVDSQHV GHSVLTAKGM ALFGEGKAEF TGRHDAHLNG

3001 KVIGTLKNSL FFSAQPFEIT ASTNNEGNLK VRFPLRLTGK IDFLNNYALF

3051 LSPSAQQASW QVSARFNQYK YNQNFSAGNN ENIMEAHVGI NGEANLDFLN

3101 IPLTIPEMRL PYTIITTPPL KDFSLWEKTG LKEFLKTTKQ SFDLSVKAQY

3151 KKNKHRHSIT NPLAVLCEFI SQSIKSFDRH FEKNRNNALD FVTKSYNETK

3201 IKFDKYKAEK SHDELPRTFQ IPGYTVPVVN VEVSPFTIEM SAFGYVFPKA

3251 VSMPSFSILG SDVRVPSYTL ILPSLELPVL HVPRNLKLSL PHFKELCTIS

3301 HIFIPAMGNI TYDFSFKSSV ITLNTNAELF NQSDIVAHLL SSSSSVIDAL

3351 QYKLEGTTRL TRKRGLKLAT ALSLSNKFVE GSHNSTVSLT TKNMEVSVAK

3401 TTKAEIPILR MNFKQELNGN TKSKPTVSSS MEFKYDFNSS MLYSTAKGAV

3451 DHKLSLESLT SYFSIESSTK GDVKGSVLSR EYSGTIASEA NTYLNSKSTR

3501 SSVKLQGTSK IDDIWNLEVK ENFAGEATLQ RIYSLWEHST KNHLQLEGLF

3551 FTNGEHTSKA TLELSPWQMS ALVQVHASQP SSFHDFPDLG QEVALNANTK

3601 NQKIRWKNEV RIHSGSFQSQ VELSNDQEKA HLDIAGSLEG HLRFLKNIIL

3651 PVYDKSLWDF LKLDVTTSIG RRQHLRVSTA FVYTKNPNGY SFSIPVKVLA

3701 DKFITPGLKL NDLNSVLVMP TFHVPFTDLQ VPSCKLDFRE IQIYKKLRTS

3751 SFALNLPTLP EVKFPEVDVL TKYSQPEDSL IPFFEITVPE SQLTVSQFTL

3801 PKSVSDGIAA LDLNAVANKI ADFELPTIIV PEQTIEIPSI KFSVPAGIVI

3851 PSFQALTARF EVDSPVYNAT WSASLKNKAD YVETVLDSTC SSTVQFLEYE

3901 LNVLGTHKIE DGTLASKTKG TLAHRDFSAE YEEDGKFEGL QEWEGKAHLN

3951 IKSPAFTDLH LRYQKDKKGI STSAASPAVG TVGMDMDEDD DFSKWNFYYS

4001 PQSSPDKKLT IFKTELRVRE SDEETQIKVN WEEEAASGLL TSLKDNVPKA

4051 TGVLYDYVNK YHWEHTGLTL REVSSKLRRN LQNNAEWVYQ GAIRQIDDID

4101 VRFQKAASGT TGTYQEWKDK AQNLYQELLT QEGQASFQGL KDNVFDGLVR

4151 VTQKFHMKVK HLIDSLIDFL NFPRFQFPGK PGIYTREELC TMFIREVGTV

4201 LSQVYSKVHN GSEILFSYFQ DLVITLPFEL RKHKLIDVIS MYRELLKDLS

4251 KEAQEVFKAI QSLKTTEVLR NLQDLLQFIF QLIEDNIKQL KEMKFTYLIN

4301 YIQDEINTIF NDYIPYVFKL LKENLCLNLH KFNEFIQNEL QEASQELQQI

4351 HQYIMALREE YFDPSIVGWT VKYYELEEKI VSLIKNLLVA LKDFHSEYIV

4401 SASNFTSQLS SQVEQFLHRN IQEYLSILTD PDGKGKEKIA ELSATAQEII

4451 KSQAIATKKI ISDYHQQFRY KLQDFSDQLS DYYEKFIAES KRLIDLSIQN

4501 YHTFLIYITE LLKKLQSTTV MNPYMKLAPG ELTIIL

CLAIMS

1. A peptide compound of formula:

Z^1-K-A-Q-X^1-K-K-N-K-H-R-H-S-X^2-T-Z^2 [SEQ ID No. 1]

where:

X^1 represents S or Y,

X^2 represents T or I,

Z^1 represents the N terminus of the peptide, or from 1 to 47 amino acids,

Z^2 represents the C terminus of the peptide, a terminal amide group, or from 1 to 77 amino acids;

or a variant of this peptide which contains one or more internal deletions, insertions or substitutions and which substantially retains anti-coagulant properties of apoB-100.

2. A peptide compound according to claim 1 whose sequence consists essentially of amino acids 21 to 118 of Seq. ID. No. 12.

3. A peptide compound comprising

(i) a first peptide which is a peptide as defined in claim 1 or 2; and, attached to said first peptide,

(ii) a second peptide, which enhances the anticoagulant activity of the first peptide and comprises a positively charged sequence from within amino acids 3300 to 3400 of apolipoprotein B-100, or a variant of this sequence which contains one or more internal deletions, insertions or substitutions.

4. A pharmaceutical composition comprising a peptide compound according to any one of claims 1 to 3 together with a pharmaceutically acceptable diluent or carrier.

5. A pharmaceutical composition comprising

(i) a first peptide which is a peptide as defined in claim 1 or 2; and

(ii) a second peptide, which enhances the anticoagulant activity of the first peptide and comprises a positively charged sequence form within

amino acids 3300 to 3400 of apolipoprotein B-100, or a variant of this sequence which contains one or more internal deletions, insertions or substitutions.

6. A compound according to any one of claims 1 to 3 or a composition according to claim 4 or 5 for use in a method of treatment or therapy of the human or animal body.

7. A composition comprising a compound according to any one of claims 1 to 3 and a matrix metalloproteinase inhibitor.

8. A product containing a peptide compound according to any one of claims 1 to 3 or a combination of peptides as defined in claim 4 and a matrix metalloproteinase inhibitor as a combined preparation for simultaneous, separate or sequential use in cancer therapy.

9. Use of a peptide compound according to any one of claims 1 to 3 or a combination of peptides as defined in claim 4 in the manufacture of a medicament for the inhibition of a thromboplastin-related process.

10. Use according to claim 9 in the manufacture of a medicament for the preventing or reducing blood coagulation.

11. Use according to claim 9 in the manufacture of a medicament for the inhibition of angiogenesis, cellular differentiation or apoptosis.

12. A product containing

(i) a first peptide which is a peptide as defined in claim 1 or 2; and

(ii) a second peptide, which enhances the anticoagulant activity of the first peptide and comprises a positively charged sequence from within amino acids 3300 to 3400 of apolipoprotein B-100 or a variant of this sequence which contains one or more internal deletions, insertions or substitutions;

as a combined preparation for simultaneous, separate or sequential use for inhibition of a thromboplastin-related process.

13. A method of treatment of a patient who requires therapy for a condition requiring inhibition of a thromboplastin-related process which comprises administering to a patient in need of treatment an effective amount of the compound or composition according to any one of claims 1 to 3 or a combination of peptides as defined in claim 4.

14. A method according to claim 12 for the treatment of a patient who requires anti-coagulant therapy.

15. A method according to claim 12 for the inhibition of angiogenesis, cellular differentiation or apoptosis.

Fig.1.

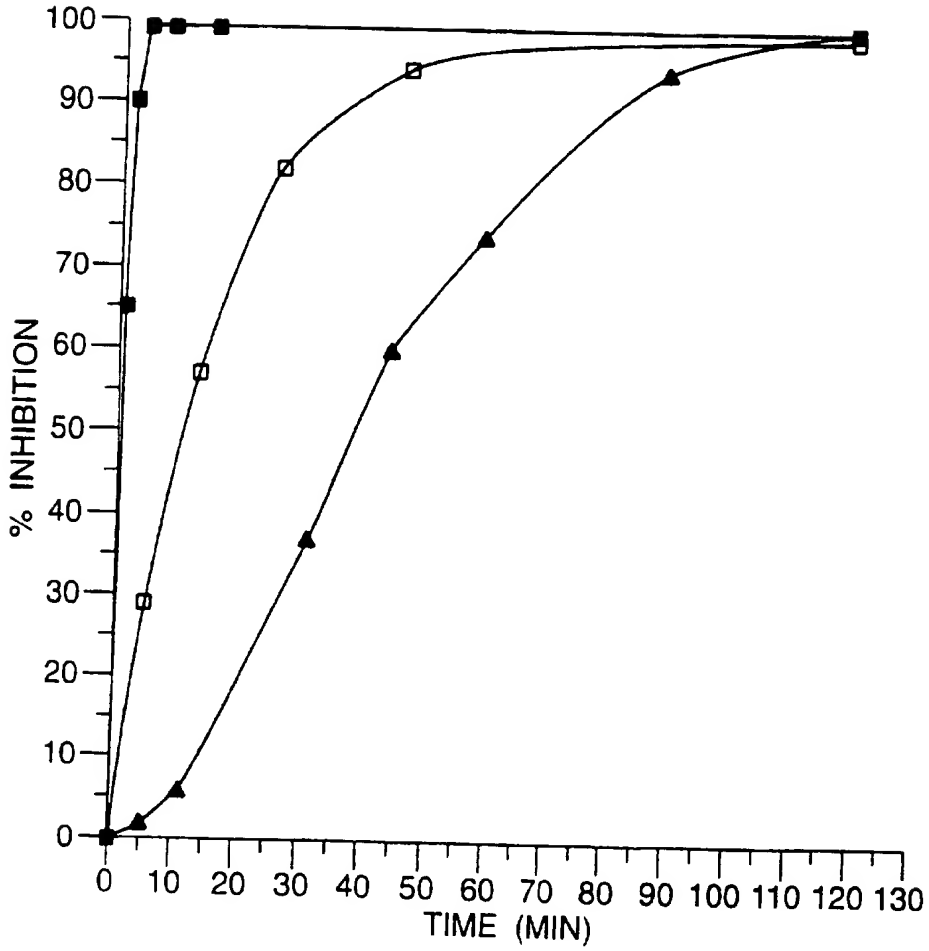

% INHIBITION
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100 110 120 130
TIME (MIN)

Fig.2.

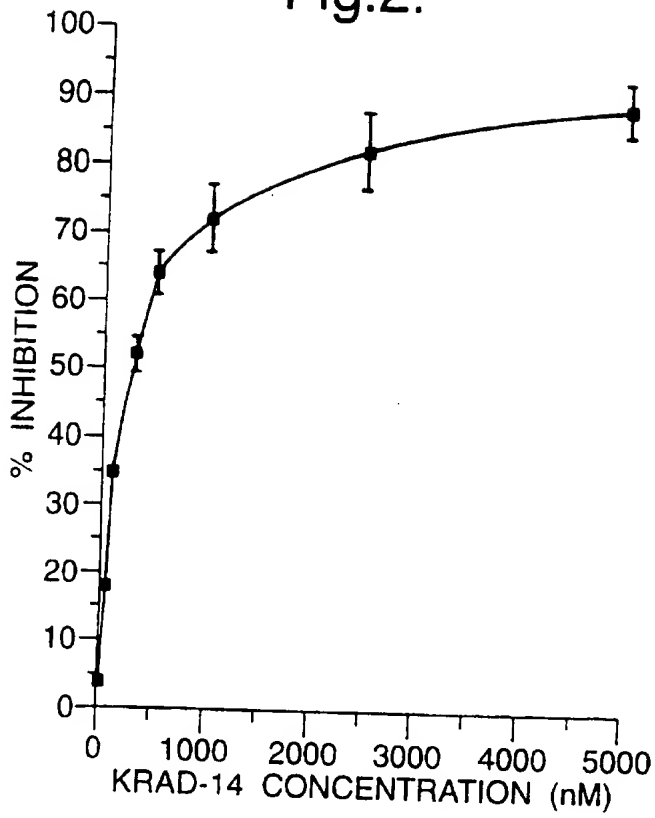

100
90
80
70
60
50
40
30
20
10
0
% INHIBITION
0
1000
2000
3000
4000
5000
KRAD-14 CONCENTRATION (nM)

Fig.3(b).

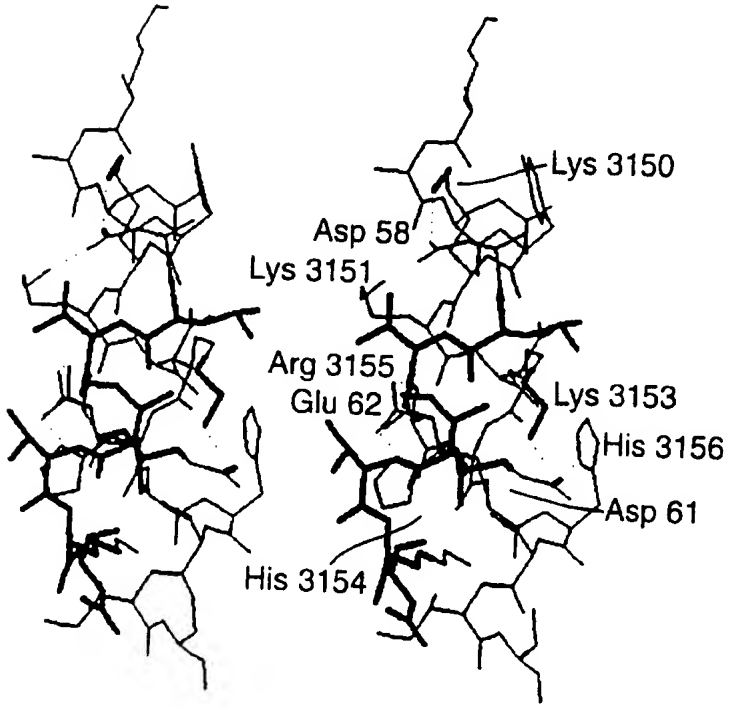
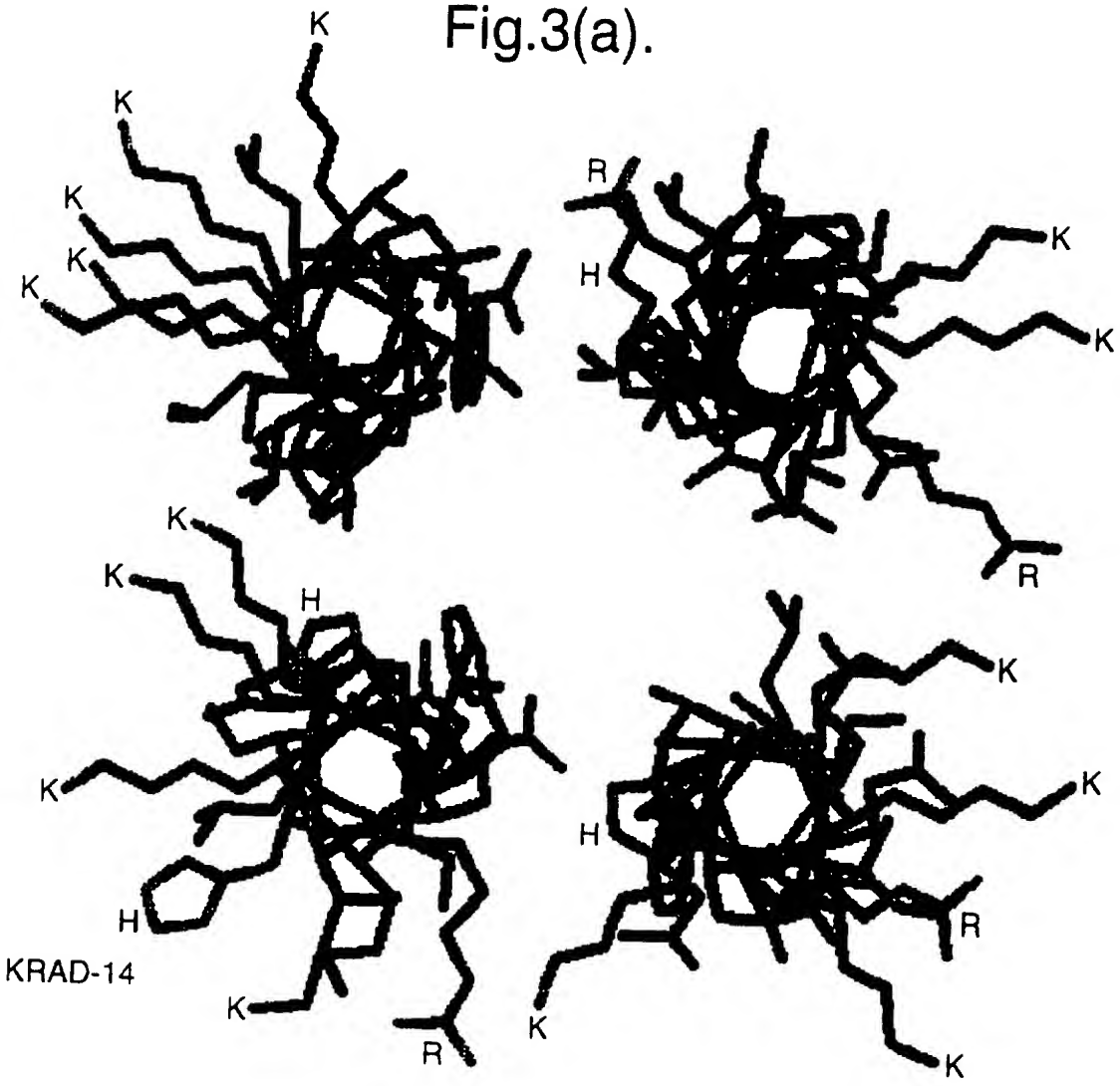

Lys 3150
Asp 58
Lys 3151
Arg 3155
Glu 62
Lys 3153
His 3156
Asp 61
His 3154

Fig.3(a).



KRAD-14

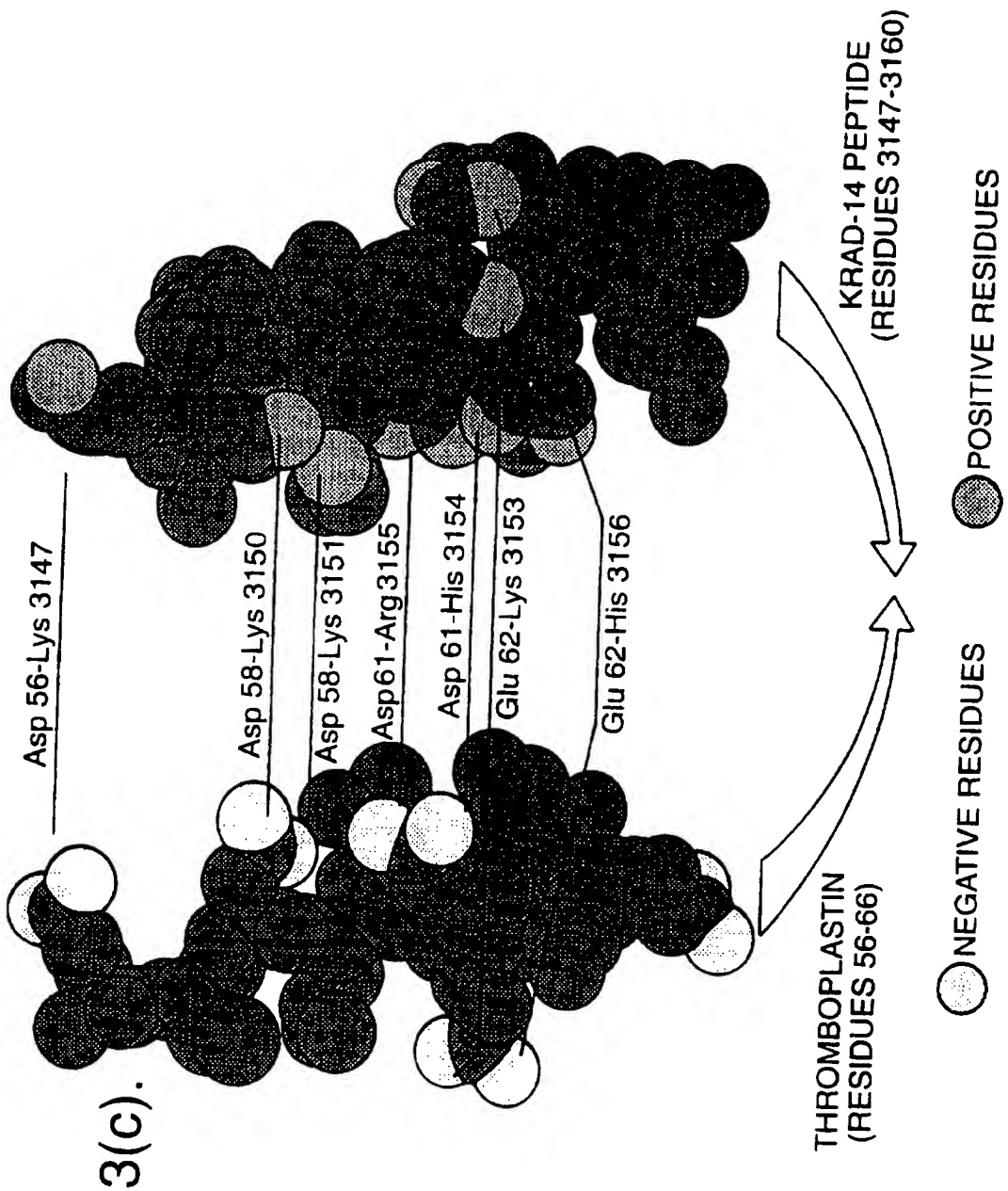

Asp 56-Lys 3147
Asp 58-Lys 3150
Asp 58-Lys 3151
Asp61-Arg3155
Asp 61-His 3154
Glu 62-Lys 3153
Glu 62-His 3156
KRAD-14 PEPTIDE
(RESIDUES 3147-3160)
POSITIVE RESIDUES
THROMBOPLASTIN
(RESIDUES 56-66)
NEGATIVE RESIDUES

Fig.3(c).

Fig.4.

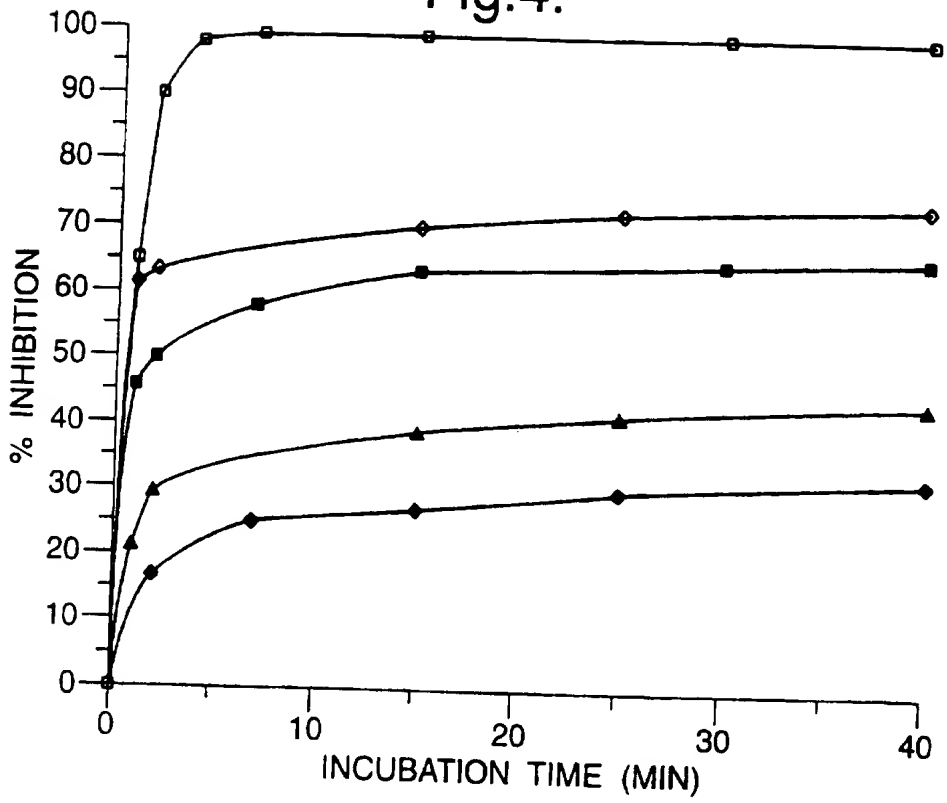

100
90
80
70
60
50
40
30
20
10
0
% INHIBITION
0
10
20
30
40
INCUBATION TIME (MIN)

Fig.5.

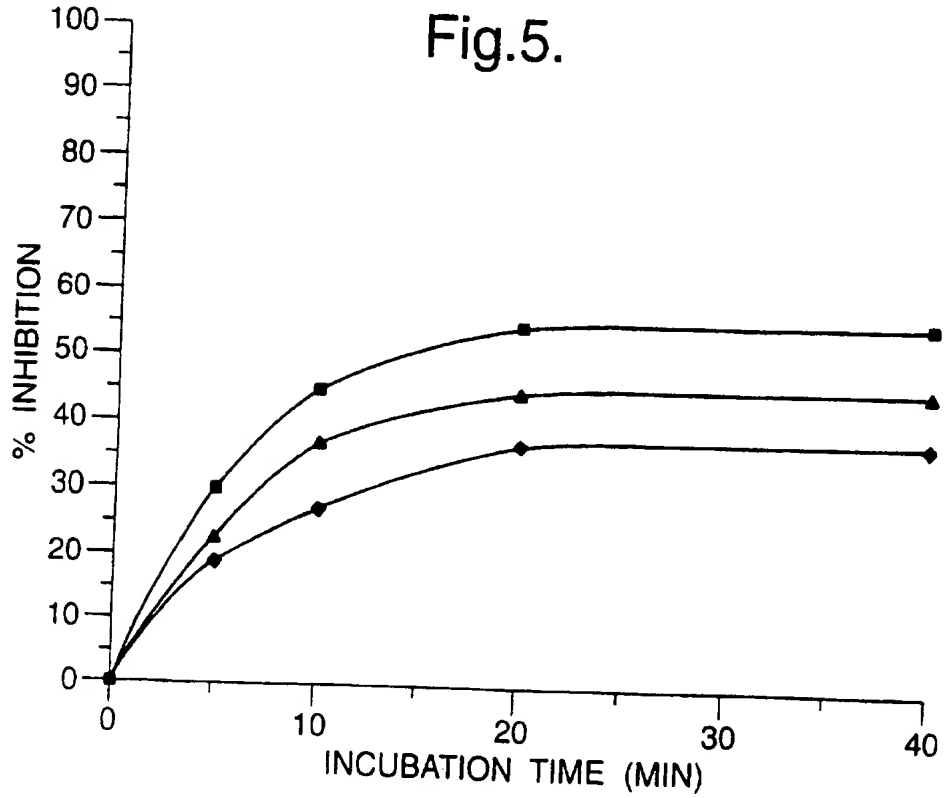
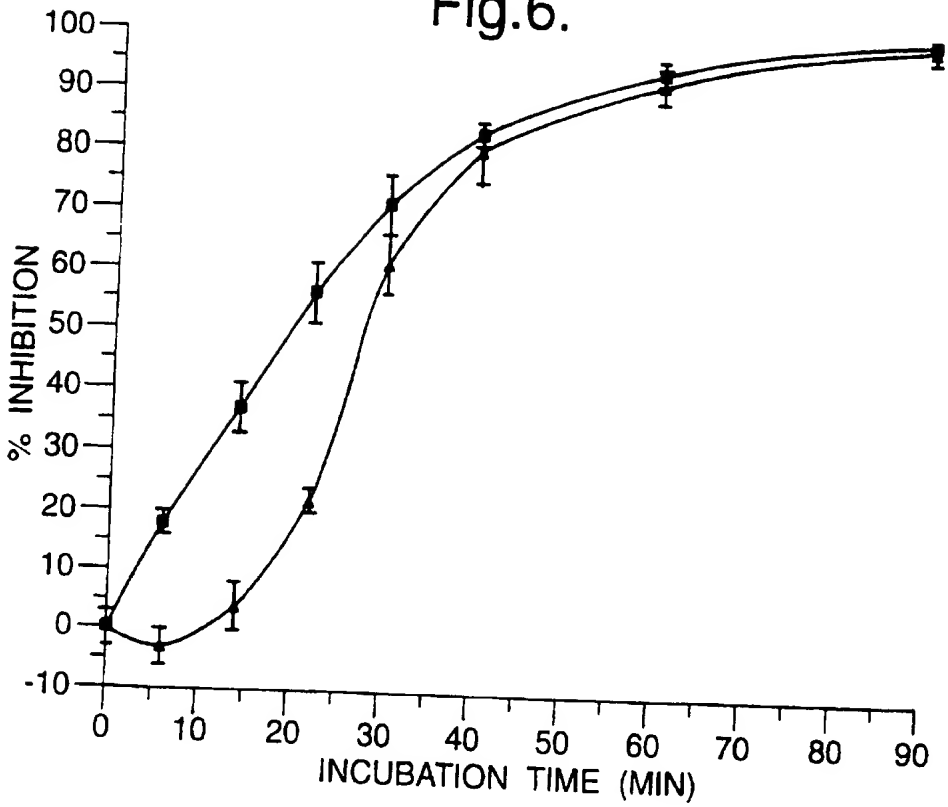

100
90
80
70
60
50
40
30
20
10
0
% INHIBITION
0
10
20
30
40
INCUBATION TIME (MIN)

Fig.6.


100
90
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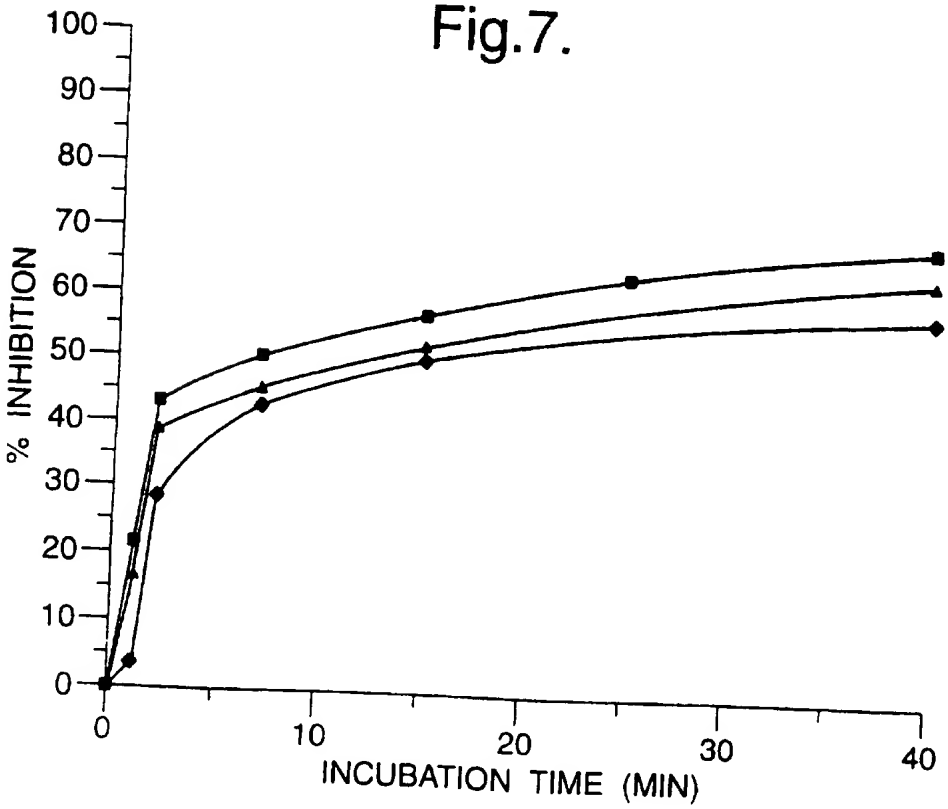
70
60
50
40
30
20
10
0
-10
% INHIBITION
0
10
20
30
40
50
60
70
80
90
INCUBATION TIME (MIN)

Fig.7.


100
90
80
70
60
50
40
30
20
10
0
% INHIBITION
0
10
20
30
40
INCUBATION TIME (MIN)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/01255

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/775 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	C. ETTELAIE AND R.M. HOWELL: "The inhibition of Thromboplastin by apolipoprotein-B and the effect of various lipids" THROMBOSIS RESEARCH, vol. 68, no. 2, 1992, US, pages 175-184, XP002040391 see page 182 --- -/--	1,4,10

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
22 September 1997	22.10.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Fuhr, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interna[illegible]al Application No
PCT/GB 97/01255

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	C. ETTELAI ET AL.: "The Mechanism of Inibition of Factor III (Thromboplastin) Activity by Apolipoprotein B-100" ARTERIOSCLEROSIS, THROMBOSIS AND VASCULAR BIOLOGY, vol. 16, no. 5, May 1996, US, pages 639-647, XP002040392 see page 646, left-hand column, paragraph 3 - page 647, left-hand column, paragraph 2 ---	1,4,10
A	C. ETTELAIE ET AL.: "The effect of lipid peroxidation and lipolysis on the ability of lipoproteins to influence thromboplastin activity" BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1257, 1995, AMSTERDAM, NL, pages 5-30, XP002041408 see page 28, right-hand column, last paragraph - page 29, right-hand column, paragraph 3 ---	1,4,10
T	C. ETTELAIE ET AL.: "Alterations in the structure of apolipoprotein B-100 determine the behaviour of LDL towards thromboplastin" BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1345, no. 3, 1997, AMSTERDAM, NL, pages 237-247, XP002040393 see page 243, right-hand column, paragraph 2 - page 244, left-hand column, paragraph 1 see page 244, right-hand column, paragraph 2 - page 246, left-hand column, paragraph 2 -----	1,4,10

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 97/01255

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 13-15
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 13-15 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.